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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : **Not classified**	A2	(11) International Publication Number: **WO 99/55132**
		(43) International Publication Date: 4 November 1999 (04.11.99)

(21) International Application Number: PCT/NL99/00235

(22) International Filing Date: 23 April 1999 (23.04.99)

(30) Priority Data:
09/065,752   24 April 1998 (24.04.98)   US

(71) Applicant: INTROGENE B.V. [NL/NL]; Wassenaarseweg 72, NL–2333 AL Leiden (NL).

(72) Inventors: VOGELS, Ronald; Van Rietlaan 4, NL–3461 HW Linschoten (NL). BOUT, Abraham; Coymansstraat 24, NL–2751 AR Moerkapelle (NL).

(74) Agent: OTTEVANGERS, S., U.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL–2587 BN The Hague (NL).

(81) Designated States: AU, CA, JP, MX, NZ.

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: GENERATION OF PACKAGING SYSTEM FOR HUMAN RECOMBINANT ADENOVIRAL VECTORS
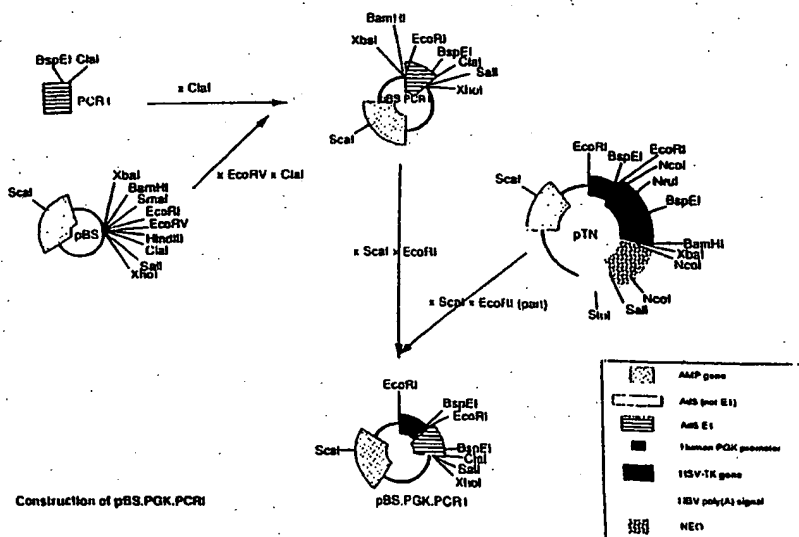
(57) Abstract

The invention discloses novel means and methods for the generation of adenovirus vector. One method of the invention entails a method for generating an adenovirus vector comprising welding together two nucleic acid molecules whereby said molecules comprise partially overlapping sequences capable of combining with each other allowing the generation of a physically linked nucleic acid comprising at least two functional adenovirus inverted terminal repeats, a functional encapsulation signal and a nucleic acid of interest or functional parts, derivatives and/or analogues thereof. The invention further discloses nucleic acid molecules for generation adenovirus vectors.

Title: Packaging systems for human recombinant adenovirus to
        be used in gene therapy

The invention relates to the field of recombinant DNA
technology, more in particular to the field of gene therapy.
In particular the invention relates to gene therapy using
materials derived from adenovirus, in particular human
5   recombinant adenovirus. It especially relates to novel virus
derived vectors and novel packaging cell lines for vectors
based on adenoviruses.

Gene therapy is a recently developed concept for
which a wide range of applications can be and have been
10   envisaged. In gene therapy a molecule carrying genetic
information is introduced into some or all cells of a host,
as a result of which the genetic information is added to the
host in a functional format. Included is the treatment of
genetic disorders by providing the genetic information for
15   supplementing a protein or other substance which is, through
said genetic disorder, not present or at least present in
insufficient amounts in the host, the treatment of tumors and
(other) acquired disease such as (auto) immune diseases or
infections, or other processes. The genetic information added
20   may be a gene or a derivative of a gene, such as a cDNA,
which encodes a protein. In this case the functional format
means that the protein can be expressed by the machinery of
the host cell. The genetic information can also be a sequence
of nucleotides complementary to a sequence of nucleotides (be
25   it DNA or RNA) present in the host cell. The functional
format in this case is that the added DNA (nucleic acid)
molecule or copies made thereof in situ are capable of base
pairing with the complementary sequence present in the host
cell.   .
30   Thus, there are basically three different approaches
in gene therapy, one directed towards compensating a
deficiency present in a (mammalian) host; the second directed

2

towards the removal or elimination of unwanted substances
(organisms or cells) and the third directed towards
application of a recombinant vaccine (tumors or foreign
microorganisms).

5   For the purpose of gene therapy, adenoviruses
carrying deletions have been proposed as suitable vehicles.
Adenoviruses are non-enveloped DNA viruses. Gene transfer
vectors derived from adenoviruses (so called adenoviral
vectors) have a number of features that make them
10   particularly useful for gene transfer for such purposes. For
example, the biology of the adenoviruses is characterized in
detail, the adenovirus is not associated with severe human
pathology, the virus is extremely efficient in introducing
its DNA into the host cell, the virus can infect a wide
15   variety of cells and has a broad host range, the virus can be
produced in large quantities with relative ease, and the
virus can be rendered replication detective by deletions in
the early region 1 (E1) of the viral genome.
          The adenovirus (Ad) genome is a linear double-
20   stranded DNA molecule of approximately 36000 base pairs with
the 55-kDa terminal protein covalently bound to the
5'terminus of each strand. The Ad DNA contains identical
Inverted Terminal Repeats (ITR) of about 100 base pairs with
the exact length depending on the serotype. The viral origins
25   of replication are located within the ITRs exactly at the
genome ends. DNA synthesis occurs in two stages. First, the
replication proceeds by strand displacement, generating a
daughter duplex molecule and a parental displaced strand. The
displaced strand is single stranded and can form a so called
30   "panhandle" intermediate, which allows replication initiation
and generation of a daughter duplex molecule. Alternatively,
replication may proceed from both ends of the genome
simultaneously, obviating the requirement to form the
panhandle structure. The replication is summarized in Figure
35   14 adapted from Lechner *et al*, (1977) *J. Mol. Biol.*
174:493-510.

During the productive infection cycle, the viral
genes are expressed in two phases: the early phase, which is
the period up to viral DNA replication, and the late phase,
which coincides with the initiation of viral DNA replication.
5   During the early phase only the early gene products, encoded
by regions E1, E2, E3 and E4, are expressed, which carry out
a number of functions that prepare the cell for synthesis of
viral structural proteins (Berk, A. J. (1986) *Ann. Rev.*
Genet. 20:45-79). During the late phase the late viral gene
10 products are expressed in addition to the early gene products
and host cell DNA and protein synthesis are shut off.
Consequently, the cell becomes dedicated to the production of
viral DNA and of viral structural proteins (Tooze, J. (1981)
DNA Tumor Viruses (revised). Cold Spring Harbor Laboratory.
15 Cold Spring Harbor, New York).
        The E1 region of adenovirus is the first region of
adenovirus expressed after infection of the target cell. This
region consists of two transcriptional units, the E1A and E1B
genes, both of which are required for oncogenic
20 transformation of primary (embryonal) rodent cultures. The
main functions of the E1A gene products are to induce
quiescent cells to enter the cell cycle and resume cellular
DNA synthesis, and to transcriptionally activate the E1B gene
and the other early regions (E2, E3 and E4) of the viral
25 genome. Transfection of primary cells with the E1A gene alone
can induce unlimited proliferation (immortalization), but
does not result in complete transformation. However,
expression of E1A in most cases results in induction of
programmed cell death (apoptosis), and only occasionally
30 immortalization is obtained (Jochemsen *et al*, (19987) *EMBO J.*
6:3399-3405). Co-expression of the E1B gene is required to
prevent induction of apoptosis and for complete morphological
transformation to occur. In established immortal cell lines,
high level expression of E1A can cause complete
35 transformation in the absence of E1B (Roberts *et al*, (1985)
J. Virol. 56:404-413).

The E1B encoded proteins assist E1A in redirecting the cellular functions to allow viral replication. The E1B 55 kD and E4 33kD proteins, which form a complex that is essentially localized in the nucleus, function in inhibiting

5   the synthesis of host proteins and in facilitating the expression of viral genes. Their main influence is to establish selective transport of viral mRNAs from the nucleus to the cytoplasm, concomitantly with the onset of the late phase of infection. The E1B 21 kD protein is important for

10 correct temporal control of the productive infection cycle, thereby preventing premature death of the host cell before the virus life cycle has been completed. Mutant viruses incapable of expressing the E1B 21 kD gene product exhibit a shortened infection cycle that is accompanied by excessive

15 degradation of host cell chromosomal DNA (*deg*-phenotype) and in an enhanced cytopathic effect (*cyt*-phenotype) (Telling *et al*, (1994) *J. Virol.* 68:541-7). The *deg* and *cyt* phenotypes are suppressed when in addition the E1A gene is mutated, indicating that these phenotypes are a function of E1A (White

20 *et al*, (1988) *J. Virol.* 62:3445-3454). Furthermore, the E1B 21 kDa protein slows down the rate by which E1A switches on the other viral genes. It is not yet known through which mechanisms E1B 21 kD quenches these E1A dependent functions. Vectors derived from human adenoviruses, in which at

25 least the E1 region has been deleted and replaced by a gene of interest, have been used extensively for gene therapy experiments in the pre-clinical and clinical phase, and all adenovirus vectors currently used in gene therapy have a deletion in the E1 region, where novel genetic information

30 can be introduced. The E1 deletion renders the recombinant virus replication defective (Stratford-Perricaudet *et al*, (1991) pp. 51-61. In O. Cohen-Adenaur, and M. Boiron (Eds): *Human Gene Transfer*, John Libbey Eurotext).

     In contrast to, for example, retroviruses,

35 adenoviruses do not integrate into the host cell genome, are

able to infect non-dividing cells and are able to efficiently transfer recombinant genes *in vivo (Brody et al, (1994) Ann NY Acad. Sci.* 716:90-101). These features make adenoviruses attractive candidates for *in vivo* gene transfer of, for example, suicide or cytokine genes into tumor cells. However, a problem associated with current recombinant adenovirus technology is the possibility of unwanted generation of replication competent adenovirus (RCA) during the production of recombinant adenovirus (Lochmüller *et al*, (1994) *Hum. Gene Ther.* 5:1485-1492; Imler *et al*, (1996) *Gene Ther.* 3:75-84). This is caused by homologous recombination between overlapping sequences from the recombinant vector and the adenovirus constructs present in the complementing cell line, such as the 293 cells (Graham *et al*, (1977) *J. Gen. Virol.* 36:59-72). RCA in batches to be used in clinical trials is unwanted because RCA i) will replicate in an uncontrolled fashion; ii) can complement replication defective recombinant adenovirus, causing uncontrolled multiplication of the recombinant adenovirus; and iii) batches containing RCA induce significant tissue damage and hence strong pathological side effects (Lochmüller *et al*, (1994) *Hum. Gene Ther.* 5:1485-1492). Therefore, batches to be used in clinical trials should be proven, free of RCA (Ostrove, J. M. (1994) *Cancer Gene Ther.* 1:125-131).

One of the additional problems associated with the use of recombinant adenovirus vectors is the host defense reaction against treatment with adenovirus. Briefly, recombinant adenoviruses are deleted for the E1 region (*see* above). The adenovirus E1 products trigger the transcription of the other early genes (E2, E3, E4), which consequently activate expression of the late virus genes. Therefore, it was generally thought that E1 deleted vectors would not express any other adenovirus genes. However, recently it has been demonstrated that some cell types are able to express adenovirus genes in the absence of E1 sequences. This

indicates, that some cell types posses the machinery to drive transcription of adenovirus genes. In particular, it was demonstrated that such cells synthesize E2A and late adenovirus proteins. In a gene therapy setting, this means
5   that transfer of the therapeutic recombinant gene to somatic cells not only results in expression of the therapeutic protein but may also result in the synthesis of viral proteins. Cells that express adenoviral proteins are recognized and killed by cytotoxic T Lymphocytes, which both
10 eradicates the transduced cells and causes inflammation (Bout et al, (1994a) Gene Therapy 1:385-394; Engelhardt et al, (1993) Human Gene Therapy 4:759-769; Simon et al, (1993) Human Gene Therapy 4:771-780). As this adverse reaction hampers gene therapy, several solutions to this problem have
15 been suggested, such as using immunosuppressive agents after treatment, retaining the adenovirus E3 region in the recombinant vector (see patent application EP 952022 1B) or using ts mutants of human adenovirus, which have a point mutation in the E2A region (patent WO/28938). However, these
20 strategies to circumvent the immune response have their limitations. The use of ts mutant recombinant adenovirus diminishes the immune response to some extent, but is less effective in preventing pathological responses in the lungs (Engelhardt et al, (1994a) Human Gene Ther. 5:1217-1229). The
25 E2A protein may induce an immune response by itself and it plays a pivotal role in the switch to the synthesis of late adenovirus proteins. Therefore, it is attractive to make recombinant adenoviruses which are mutated in the E2 region, rendering it temperature sensitive (ts), as has been claimed
30 in patent application WO/28938. A major drawback of this system is the fact that, although the E2 protein is unstable at the non-permissive temperature, the immunogenic protein is still synthesized. In addition, it is to be expected that the unstable protein does activate late gene expression, albeit
35 to a low extent. ts125 mutant recombinant adenoviruses have been tested, and prolonged recombinant gene expression was

reported (Yang et al, (1994b) Nat Genet. 7:362-369;
Engelhardt et al, (1994a) Hum. Gene Ther. 5:1217-1229;
Engelhardt et al, (1994b) Proc. Natl. Acad. Sci USA 91:6196-
200; Yang et al, (1995) J. Virol. 69:2004-2015). However,

5   pathology in the lungs of cotton rats was still high
(Engelhardt et al, (1994a) Human Gene Ther. 5:1217-1229),
indicating that the use of ts mutants results in only a
partial improvement in recombinant adenovirus technology.
Others (Fang et al, (1996) Gene Ther. 3:217-222) did not

10 observe prolonged gene expression in mice and dogs using
ts125 recombinant adenovirus. An additional difficulty
associated with the use of ts125 mutant adenoviruses is that
a high frequency of reversion is observed. These revertants
are either real revertants or the result of second site

15 mutations (Kruijer et al, (1983) Virology 124:425-433;
Nicolas et al, (1981) Virology 108:521-524). Both types of
revertants have an E2A protein that functions at normal
temperature and therefore have similar toxicity as the wild-
type virus.

20   E1 deleted recombinant adenoviruses are usually made
by one of the following method. In the first method,
adenovirus DNA, be it wild type (wt) or E1 and/or E3 deleted,
is digested with a restriction enzyme e.g. ClaI, to remove
the left ITR, packaging signal and at least part of the E1

25 sequences and the remaining adenovirus genome fragment (1) is
purified. Cotransfection of (1) with a linearized adapter
construct (2) containing the left ITR, packaging signal, an
expression cassette with the gene of interest and adenovirus
sequences overlapping with (1) in a cell line complementing

30 for E1 functions (packaging cell line) will give rise to
recombinant adenovirus particles by intra-cellular homologous
recombination. Alternatively, an adapter construct (3)
containing the left ITR, packaging signal, and an expression
cassette with the gene of interest is such that it can be

35 ligated to the adenovirus DNA fragment (1) followed by

transfection into packaging cells. The disadvantage of these methods is that the purification of (1) is laborious and that incomplete digestion of wt DNA results in introduction of wt adenovirus into the culture leading to contamination. An

5   approach to circumvent this problem has been by the construction of clone pHBG10 described by Bett *et al*, (1994) *Natl. Acad. Sci. USA* 91:8802-8806. This plasmid clone contains Ad5 sequences with a deletion of the packaging signal and part of the E1 region and with the viral ITRs

10 attached to each other. However, this clone comprises adenovirus sequences that are also present in E1-complementing cell lines, including those of the present invention *(see* EP 95201611.1). Furthermore, since the ITRs are attached to each other the clone cannot be linearized,

15 resulting in less efficient recombination with the E1 substitution plasmid.

In the second method, the recombinant adenoviruses is constructed either by homologous recombination in bacteria (Chartier *et al*, (1996) *J. Virol.* 70, No. 7:4805-4810;

20 Crouzet *et al*, (1997) *Proc. Natl. Acad Sci USA* 94:1414-1419) or by cloning into cosmid vectors (Fu *et al*, (1997) *Hum. Gene Ther.* 8:1321-1330) and subsequent transfection into an E1 complementing cell line. The disadvantage of this method is that it demands extensive analysis of each generated clone

25 (~35 kb) by restriction enzyme digestion before transfection to exclude deletions that occurred due to recombination in the bacteria. In addition, the use of cloned adenovirus sequences does not solve the problem of sequence overlap between commonly used packaging cells and recombinant viruses

30 leading to production of RCA during propagation.

A third method that is used is a two-step gene replacement technique in yeast, starting with a complete adenovirus genome (Ad2; Ketner *et al*, (1994) *Proc. Natl. Acad. Sci. USA* 91:6186-6190) cloned in a Yeast Artificial

35 Chromosome (YAC) and a plasmid containing adeno sequences to

target a specific region in the YAC clone, an expression
cassette for the gene of interest and a positive and negative
selectable marker. This method requires yeast technology and
extensive analysis of each new recombinant clone (even more
5   cumbersome than the above described method, due to the large
size of YACS).

A fourth method uses a cosmid clone (pAdex1w; Miyake
et al, (1996) *Medical Sciences* 93:1320-1324) that carries the
Ad5 sequence with deletions in the E1 and E3 sequences. This
10   clone has a unique restriction site replacing part of the E1
region that allows insertion of a foreign expression
cassette. For the generation of recombinant adenoviruses, a
DNA-terminal protein complex (DNA-TPC) is isolated from cells
infected with a replication competent adenovirus Ad-dlX (wt
15   Ad5 with an *XbaI* deletion in the E3 region). This DNA is
digested with *EcoT22I* to remove the 5' part of the DNA and
cotransfected with the cosmid cloned into E1 complementing
cells. Intracellular recombination generates the recombinant
virus (Miyake *et al*, (1996) *Medical Sciences* 93:1320-1324).
20   This method has the disadvantage that replication competent
viral DNA is used and that the E1 deletion in the cosmid
clone is not enough to remove all overlap with E1 sequences
in currently used packaging cell lines including those used
in the present invention. Thus, current methods to generate
25   RCA-free recombinant adenoviruses have several disadvantages,
including the risk of introducing wild-type viruses in the
culture, instability of cloned adenovirus sequences, the
necessity to check the complete ~35 kb recombinant clone by
restriction analysis for each new virus to be generated, and
30   the system being suitable only for E1 deleted recombinant
adenoviruses and much more laborious for use with recombinant
adenoviruses comprising E3 substitutions. Furthermore,
despite the use of cloned adenovirus DNA in some of the
methods, extensive overlap with adenovirus sequences present
35   in commonly used packaging cells like 293 and 911 cells does
not solve the problem of appearance of RCA due to homologous

recombination during propagation of the virus. Therefore, a
need persists for methods and means to produce RCA-free
recombinant adenovirus preparations that solve the
disadvantages of prior art methods and means discussed above.
5 Gene addition is currently by far the most widely applied
gene therapy technique. This is mainly due to the fact that
a) homologous recombination is very inefficient and b) for
homologous recombination relatively large DNA fragments are
required for which no suitable vector systems were available.
10 Thus there is currently an unmet need for vector systems that
efficiently introduce large nucleic acid molecules into
mammalian cells.

Recombinant adenoviruses are able to efficiently
15 transfer recombinant genes to the rat liver and airway
epithelium of rhesus monkeys (Bout et al, (1994b) *Human Gene
Therapy* 5:3-10; Bout et al, (1994a) *Gene Therapy* 1:385-394).
In addition, (Vincent et al, (1996) *J. Neurosurg* 85:648-654;
Vincent et al, (1996b) *Hum. Gene Ther.* 7:197-205) and others
20 (see for example Haddada et al, (1993) *Hum. Gene Ther.* 4:703-
11) have observed an efficient *in vivo* adenovirus mediated
gene transfer to a variety of tumor cells *in vitro* and to
solid tumors in animal models (lung tumors, glioma) and human
xenografts in immunodeficient mice (lung) *in vivo* (reviewed
25 by Blaese et al, *Cancer Gene Ther.* 2:291-297).

Generation of minimal adenovirus vectors has been
disclosed in WO 94/12649. The method described exploits the
function of the protein IX for the packaging of minimal
adenovirus vectors (Pseudo Adenoviral Vectors (PAV) in the
30 terminology of WO 94/12649). PAVs are produced by cloning an
expression plasmid with the gene of interest between the
left-hand (including the sequences required for
encapsulation) and the right-hand adenoviral ITRs. The PAV is
propagated in the presence of a helper virus. Encapsidation
35 of the PAV is preferred compared to the helper virus because

the helper virus is partially defective for packaging (either
by virtue of mutations in the packaging signal or by virtue
of its size (virus genomes greater than 37.5 kb package
inefficiently)). In addition, the authors propose that in the
5   absence of the protein IX gene the PAV will be preferentially
packaged. However, neither of these mechanisms appear to be
sufficiently restrictive to allow packaging of only
PAVs/minimal vectors. The mutations proposed in the packaging
signal diminish packaging, but do not provide an absolute
10   block as the same packaging activity is required to propagate
the helper virus. Also neither an increase in the size of the
helper virus nor the mutation of the protein IX gene will
ensure that PAV is packaged exclusively. Thus, the method
described in WO 94/12649 is unlikely to be useful for the
15   production of helper-free stocks of minimal adenovirus
vectors/PAVs.

        Novel compositions and methods are provided for
producing recombinant adenoviruses, not only E1-deleted but
20   also minimal adenoviruses which are free of replication
competent adenoviruses. The compositions include constructs
suitable for the generation of double insert viruses. The
system provided by the invention to generate for example E1
deleted adenoviruses consists of two nucleic acid molecules
25   of which a first is a relatively small and easy to manipulate
adapter plasmid containing at least, in an operable
configuration, the left ITR, the packaging signal, if desired
an expression cassette with the nucleic acid molecule of
interest and sequences homologous to a part of a second
30   molecule that comprises at least one partially overlapping
nucleic acid molecule that comprise at least the right ITR
and preferably further comprises adenovirus sequences
encoding adenovirus capsid proteins ; and the packaging cells
of the invention described *infra*. Co-transfection of said
35   nucleic acid molecules into the packaging cells allows the
welding together of said nucleic acid molecules preferably

through essentially one homologous recombination between the overlapping sequences in said nucleic acid molecules. Homologous recombination generates a recombinant viral DNA that is able to replicate and propagate on the packaging
5   cells. The nucleic acid molecules preferably have no sequence overlap with complementing sequences in packaging cells that can lead to the formation of replication competent adenovirus (RCA). Preferably, at least one of the ITRs on the nucleic acid molecules is flanked by a restriction enzyme recognition
10 site not present in the adenoviral sequences so that the ITR can be made essentially free from vector sequences by digestion of the DNA with that restriction enzyme. In this way initiation of replication occurs more efficiently. The system provided by the present invention also greatly
15 facilitates the production of RCA-free recombinant adenoviruses with further modifications in the adenovirus genome, including but not limited to modifications in the coding regions for the E4 region proteins, hexon, penton base protein or fiber protein or E2A protein.

20

        The following embodiments should be understood to be read in the light of the combination of adapter plasmid and the large nucleic acid to be welded together therewith.

25   In one aspect of the invention the problem with RCA production is solved in that we have developed packaging cells that have no overlapping sequences with a new basic vector and thus are suited for safe large scale production of recombinant adenoviruses.
30   In another aspect of the present invention we therefore delete E2A coding sequences from the recombinant adenovirus genome and transfect these E2A sequences into the (packaging) cell lines containing E1 sequences to complement recombinant adenovirus vectors.

Major hurdles in this approach are a) that E2A should be expressed to very high levels and b) that E2A protein is very toxic to cells.

5   The current invention in yet another aspect therefore discloses use of the ts125 mutant E2A gene, which produces a protein that is not able to bind DNA sequences at the non permissive temperature. High levels of this protein may be maintained in the cells (because it is not toxic at this temperature) until the switch to the permissive temperature

10 is made. This can be combined with placing the mutant E2A gene under the direction of an inducible promoter, such as for instance tet, methallothionein, steroid inducible promoter, retinoic acid β-receptor or other inducible systems. However, in yet another aspect of the invention, the

15 use of an inducible promoter to control the moment of production of toxic wild-type E2A is disclosed.

Two salient additional advantages of E2A-deleted recombinant adenovirus are the increased capacity to harbor heterologous sequences and the permanent selection for cells

20 that express the mutant E2A. This second advantage relates to the high frequency of reversion of ts125 mutation: when reversion occurs in a cell line harboring ts125 E2A, this will be lethal to the cell. Therefore, there is a permanent selection for those cells that express the ts125 mutant E2A

25 protein. In addition, as we in one aspect of the invention generate E2A-deleted recombinant adenovirus, we will not have the problem of reversion in our adenoviruses.

In yet another aspect of the invention as a further improvement the use of non-human cell lines as packaging cell

30 lines is disclosed.

For GMP production of clinical batches of recombinant viruses it is desirable to use a cell line that has been used widely for production of other biotechnology products. Most of the latter cell lines are from monkey origin, which have

35 been used to produce, for example vaccines.

These cells cannot be used directly for the production of recombinant human adenovirus, as human adenovirus cannot replicate in cells of monkey origin or only replicates at low levels. A block in the switch of early to late phase of adenovirus lytic cycle underlies the defective replication. However, host range (hr) mutations in the human adenovirus genome are described (hr400 - 404) which allow replication of human viruses in monkey cells. These mutations reside in the gene encoding E2A protein (Klessig *et al*, (1979) *Cell* 17:957-966; Klessig *et al*, (1984) *Virus Res.* 1:169-188; Rice *et al*, (1985) *J. Virol.* 56:767-778) (Klessig *et al*, (1984) *Virus Res.* 1:169-188). Moreover, mutant viruses have been described that harbor both the hr and temperature-sensitive ts125 phenotype (Brough *et al*, (1985) *J. Virol.* 55, 206-212; Rice *et al*, (1985) *J. Virol.* 56:767-778).

We therefore generate packaging cell lines of monkey origin (e.g., VERO, CV1) that harbor:

a) E1 sequences, to allow replication of E1/E2 defective adenoviruses, and

b) E2A sequences, containing the hr mutation and the ts125 mutation named ts400 (Brough *et al*, (1985) *J. Virol.* 55:206-212; Rice *et al*, (1985) *J. Virol.*56:767-778 to prevent cell death by E2A overexpression, and/or

c) E2A sequences, just containing the hr mutation, under the control of an inducible promoter, and/or

d) E2A sequences, containing the hr mutation and the ts125 mutation (ts400), under the control of an inducible promoter.

Furthermore, we disclose the construction of novel and improved combinations of (novel and improved) packaging cell lines and (novel and improved) recombinant adenovirus vectors.

We provide:

1)   A novel packaging cell line derived from diploid
human embryonic retinoblasts (HER) that harbors nt.80-5788 of
the Ad5 genome. This cell line, named 911, deposited under
No. 95062101 at the ECACC, has many characteristics that make
5   it superior to the commonly used 293 cells (Fallaux *et al,*
(1996) *Hum. Gene Ther.* 7:215-222).

2)   Novel packaging cell lines that express just E1A
genes and not E1B genes. Established cell lines (and not
human diploid cells of which 293 and 911 cells are derived)
10   are able to express E1A to high levels without undergoing
apoptotic cell death, as occurs in human diploid cells that
express E1A in the absence of E1B. Such cell lines are able
to transcomplement E1B-defective recombinant adenoviruses,
because viruses mutated for E1B 21 kD protein are able to
15   complete viral replication even faster than wild-type
adenoviruses (Telling *et al,* (1994) *J. Virol.* 68:541-7). The
constructs are described in detail below, and graphically
represented in Figures 1-5. The constructs are transfected
into the different established cell lines and are selected
20   for high expression of E1A. This is done by operatively
linking a selectable marker gene (e.g. NEO gene) directly to
the E1B promoter. The E1B promoter is transcriptionally
activated by the EIA gene product and therefore resistance to
the selective agent (e.g. G418 in the case NEO is used as the
25   selection marker) results in direct selection for desired
expression of the E1A gene.

3)   Packaging constructs that are mutated or deleted for
E1B 21 kD, but just express the 55 kD protein.

4)   Packaging constructs to be used for generation of
30   complementing packaging cell lines from diploid cells (not
exclusively of human origin) without the need of selection
with marker genes. These cells are immortalized by expression
of E1A. However, in this particular case expression of E1B is
essential to prevent apoptosis induced by E1A proteins.
35   Selection of E1 expressing cells is achieved by selection for
focus formation (immortalization), as described for 293 cells

(Graham et al, (1977) J. Gen. Virol. 36:59-72) and 911 cells
(Fallaux et al, (1996) Hum. Gene Ther. 7:215-222), that are
E1 transformed human embryonic kidney (HEK) cells and
humanembryonic retinoblasts (HER), respectively.

5   5)   After transfection of HER cells with construct
pIG.E1A.E1B (Fig. 4), seven independent cell lines could be
established. These cell lines were designated PER.C1, PER.C3,
PER.C4, PER.C5, PER.C6, PER.C8 and PER.C9. PER denotes PGK-
E1-Retinoblasts. These cell lines express E1A and E1B

10   proteins, are stable (e.g. PER.C6 for more than 57 passages)
and complement E1-defective adenovirus vectors. Yields of
recombinant adenovirus obtained on PER cells are a little
higher than obtained on 293 cells. One of these cell lines
(PER.C6) has been deposited at the ECACC under number

15   96022940.
     6)   New adenovirus vectors with extended E1 deletions
(deletion nt.459-3510). Those viral vectors lack sequences
homologous to E1 sequences in said packaging cell lines.
These adenoviral vectors contain pIX promoter sequences and

20   the pIX gene, as pIX (from its natural promoter sequences)
can only be expressed from the vector and not by packaging
cells (Matsui et al, (1986) Mol. Cell Biol. 6:4149-4154,
Hoeben and Fallaux, pers. comm.; Imler et al, (1996) Gene
Ther. 3:75-84).

25   7)   E2A expressing packaging cell lines preferably based
on either E1A expressing established cell lines or E1A + E1B
expressing diploid cells (see under 2-4). E2A expression is
either under the control of an inducible promoter or the E2A
ts125 mutant is driven by either an inducible or a

30   constitutive promoter.
     8)   Recombinant adenovirus vectors as described before
(see 6) but carrying an additional deletion of E2A sequences.
     9)   Adenovirus packaging cells from monkey origin that
are able to transcomplement E1-defective recombinant

35   adenoviruses. They are preferably co-transfected with

pIG.E1A.E1B and pIG.NEO, and selected for NEO resistance.
Such cells expressing E1A and E1B are able to transcomplement
E1 defective recombinant human adenoviruses, but will do so
inefficiently because of a block of the synthesis of late
adenovirus proteins in cells of monkey origin (Klessig *et al*,
(1979) *Cell* 17:957-966). To overcome this problem, we
generate recombinant adenoviruses that harbor a host-range
mutation in the E2A gene, allowing human adenoviruses to
replicate in monkey cells. Such viruses are generated as
described in Figure 12., except DNA from a hr-mutant is used
for homologous recombination.
10)   Adenovirus packaging cells from monkey origin as
described under 9, except that they will also be co-
transfected with E2A sequences harboring the hr mutation.
This allows replication of human adenoviruses lacking E1 and
E2A *(see* under 8). E2A in these cell lines is either under
the control of an inducible promoter or the tsE2A mutant is
used. In the latter case, the E2A gene will thus carry both
the ts mutation and the hr mutation (derived from ts400).
Replication competent human adenoviruses have been described
that harbor both mutations (Brough *et al*, *J. Virol.* 55:206-
212; Rice *et al*, (1985) *J. Virol.* 56:767-778).

Furthermore, the present invention in one aspect
provides new cosmid and plasmid vectors containing large
fragments of the adenovirus genome and an improved method for
the generation of recombinant adenoviral vectors by making
use of these cloned adenovirus sequences.

Accordingly, the present invention provides a new
system to generate recombinant adenoviruses that is fast,
highly flexible, reliable and only requires standard cloning
technology. The new system is surprisingly efficient in
generating recombinant adenoviruses. In combination with the
packaging cells of the invention it ensures RCA-free
generation and propagation of recombinant adenoviruses. The
above listed problems associated with current methods to
generate recombinant adenoviruses are in one aspect solved by

using a functional combination of cloned adenovirus sequences
and an intra-cellular homologous recombination in suitable
packaging cells.

Accordingly, the present invention provides methods
5   and means to efficiently generate and produce vectors that
are able to harbor very large fragments of (genomic) DNA.
Vectors of the invention can be safely produced to very high
titers and are able to transduce mammalian cells, including
human cells, with high efficiency, thereby favoring
10 homologous recombination with (genomic) DNA molecules present
in said mammalian cells, due to the high numbers of
introduced DNA molecules and their large homologous overlap
with the target DNA molecules for recombination. In one
aspect vectors according to the invention are based on
15 adenoviral vectors derived from an adenoviral genome, from
which as much as possible of the adenoviral genome is deleted
except for the ITR sequences and the sequences needed *in cis*
for packaging (minimal adenovirus vectors). Such vectors can
accommodate up to 38 kb of foreign (genomic) DNA.
20   Minimal adenoviral vectors with large genomic
sequences functioning as gene replacement vectors can be
generated efficiently using the plasmid-based intracellular
PCR system disclosed *infra*, thereby avoiding the need of
contaminating helper viruses. In addition, we disclose an
25 alternative way of producing minimal adenoviral vectors
without the need for helper viruses. Replication and
packaging of the minimal adenoviral vectors with large
inserts can also be achieved by using them in combination
with a complementing molecule containing all parts of the
30 adenovirus genome that are required for replication and
packaging except for the packaging signal and E1 sequences.
Such a complementing molecule not necessarily replicates by
the virtue of the adenoviral replication machinery. It may,
for example, be cloned on a plasmid that also contains the
35 SV40 origin of replication. Transfection of this DNA together
with the minimal adenoviral vector in a E1-containing

packaging cell that also (inducibly) expresses the SV40 Large
T protein will lead to replication of the adenovirus molecule
and expression of adenoviral proteins. The latter will then
initiate replication and packaging of the minimal adenoviral
5   vectors.

A further aspect of the invention provides otherwise
improved adenovirus vectors, as well as novel strategies for
generation and application of such vectors and a method for
the intracellular amplification of linear DNA fragments in
10   mammalian cells.

The so-called "minimal" adenovirus vectors according
to the present invention retain at least a portion of the
viral genome that is required for encapsulation of the genome
into virus particles (the encapsulation signal), as well as
15   at least one copy of at least a functional part or a
derivative of the Inverted Terminal Repeat (ITR), that is DNA
sequences derived from the termini of the linear adenovirus
genome. The vectors according to the present invention
typically also contain a transgene linked to a promoter
20   sequence to govern expression of the transgene. Packaging of
the so-called minimal adenovirus vector can be achieved by
co-infection with a helper virus or, alternatively, with a
packaging deficient replicating helper system as described
below.

25   Adenovirus-derived DNA fragments that can replicate
in suitable cell lines and that may serve as a packaging
deficient replicating helper system are generated as follows.
These DNA fragments retain at least a portion of the
transcribed region of the "late" transcription unit of the
30   adenovirus genome and carry deletions in at least a portion
of the E1 region and deletions in at least a portion of the
encapsulation signal. In addition, these DNA fragments
contain at least one copy of an inverted terminal repeat
(IRT). At one terminus of the transfected DNA molecule an ITR
35   is located. The other end may contain an ITR, or
alternatively, a DNA sequence that is complementary to a

portion of the same strand of the DNA molecule other than the
ITR. If, in the latter case, the two complementary sequences
anneal, the free 3'-hydroxyl group of the 3'terminal
nucleotide of the hairpin structure can serve as a primer for
5   DNA synthesis by cellular and/or adenovirus-encoded DNA
polymerases, resulting in conversion into a double-stranded
form of at least a portion of the DNA molecule. Further
replication initiating at the ITR will result in a linear
double-stranded DNA molecule, that is flanked by two ITR's,
10 and is larger than the original transfected DNA molecule (see
Fig. 13). This molecule can replicate itself in the
transfected cell by virtue of the adenovirus proteins encoded
by the DNA molecule and the adenoviral and cellular proteins
encoded by genes in the host cell genome. This DNA molecule
15 cannot be encapsulated due to its large size (greater than
39000 base pairs) and/or due to the absence of a functional
encapsulation signal. This DNA molecule is intended to serve
as a helper for the production of defective adenovirus
vectors in suitable cell lines.
20   The invention also comprises a method for the
amplification of linear DNA fragments of variable size in
suitable mammalian cells. These DNA fragments contain at
least one copy of the ITR at one of the termini of the
fragment. As described above, the other end may contain an
25 ITR, or alternatively, a DNA sequence that is complementary
to a portion of the same strand of the DNA molecule other
than the ITR. If, in the latter case, the two complementary
sequences anneal, the free 3'-hydroxyl group of the
3'terminal nucleotide of the hairpin-structure can serve as a
30 primer for DNA synthesis by cellular and/or adenovirus-
encoded DNA polymerases, resulting in conversion of the
displaced strand into a double stranded form of at least a
portion of the DNA molecule. Further replication initiation
at the ITR will result in a linear double-stranded DNA
35 molecule, that is flanked by two ITR's, which is larger than
the original transfected DNA molecule. A DNA molecule that

contains ITR sequences at both ends can replicate itself in
transfected cells by virtue of the presence of at least the
adenovirus E2 proteins (namely the DNA binding protein (DBP),
the adenovirus DNA polymerase (Ad-pol), and the preterminal
5   protein (pTP)). The required proteins may be expressed from
adenovirus genes on the DNA molecule itself, from adenovirus
E2 genes integrated in the host-cell genome, or from a
replicating helper fragment as described above.

        Several groups have shown that the presence of ITR
10   sequences at the end of DNA molecules are sufficient to
generate adenovirus minichromosomes that can replicate, if
the adenovirus-proteins required for replication are provided
in trans e.g. by infection with a helper virus (Hu et al,
(1992) Gene 110:145-150); (Wang et al, (1985) in vivo. Nucl.
15   Acids Res. 13:5173-5187); Hay et al, (1984) J. Mol. Biol.
174:493-510). Hu et al, (1992) Gene 110:145-150, observed the
presence and replication of symmetrical adenovirus
minichromosome-dimers after transfection of plasmids
containing a single ITR. The authors were able to demonstrate
20   that these dimeric minichromosomes arise after tail-to-tail
ligation of the single ITR DNA molecules. In DNA extracted
from defective adenovirus type 2 particles, dimeric molecules
of various sizes have also been observed using electron-
microscopy (Daniell (1976) J. Virol. 19:685-708). It was
25   suggested that the incomplete genomes were formed by
illegitimate recombination between different molecules and
that variations in the position of the sequence at which the
illegitimate base pairing occurred were responsible for the
heterogeneous nature of the incomplete genomes. Based on this
30   mechanism it was speculated that, in theory, defective
molecules with a total length of up to two times the normal
genome could be generated. Such molecules could contain
duplicated sequences from either end of the genome. However,
no DNA molecules larger than the full-length virus were found
35   packaged in the defective particles (Daniell (1976) J. Virol.
19:685-708. This can be explained by the size-limitations

that apply to the packaging. In addition. it was observed
that in the virus particles DNA-molecules with a duplicated
left-end predominated over those containing the right-end
terminus (Daniell (1976) *J. Virol.* 19:685-708). This is fully

5   explained by the presence of the encapsulation signal near
that left-end of the genome (Gräble *et al*, (1990) *J. Virol.*
64:2047-2056; Gräble *et al*, (1992) *J. Virol.* 66:723-731;
Hearing *et al*, (1987) *J. Virol.* 61:2555-2558).

The major problems associated with the current

10 adenovirus-derived vectors are:
a) The strong immunogenicity of the virus particle.
b) The expression of adenovirus genes that reside in the
   adenoviral vectors, resulting in a Cytotoxic T-cell
   response against the transduced cells.

15 c) The low amount of heterologous sequences that can be
   accommodated in the current vectors (up to maximally
   approx. 8000 bp. of heterologous DNA).
d) The poor frequency and poor reliability of the methods and
   means for the generation of new adenovirus vectors.

20   Ad A) The strong immunogenicity of the adenovirus
particle results in an immunological response of the host,
even after a single administration of the adenoviral vector.
As a result of the development of neutralizing antibodies, a
subsequent administration of the virus will be less effective

25 or even completely ineffective. However, a prolonged or
persistent expression of the transferred genes will reduce
the number of administrations required and may bypass the
problem.

Ad B) Experiments performed by Wilson and

30 collaborators have demonstrated that after adenovirus-
mediated gene transfer into immunocompetent animals, the
expression of the transgene gradually decreases and
disappears approximately 2-4 weeks post-infection (Yang et
al, (1994a) *Proc. Natl. Acad. Sci USA* 91:4407-11; Yang *et al*,

35 (1994b) *Nat. Genet.* 7:362-369). This is caused by the
development of a cytotoxic T-cell (CTL) response against the

transduced cells. The CTLs were directed against adenovirus
proteins expressed by the viral vectors. In the transduced
cells synthesis of the adenovirus DNA-binding protein (the
E2A-gene product), penton and fiber proteins (late-gene

5   products) could be established. These adenovirus proteins,
encoded by the viral vector, were expressed despite deletion
of the E1 region. This demonstrates that deletion of the E1
region is not sufficient to completely prevent expression of
the viral genes (Engelhardt et al, (1994a) Human Gene Ther.

10 5:1217-1229).

        Ad C) Studies by Graham and collaborators have
demonstrated that adenoviruses are capable of encapsulating
DNA of up to 105% of the normal genome size (Ben et al,
(1993) J. Virol. 67:5911-5921). Larger genomes tend to be

15 unstable resulting in loss of DNA sequences during
propagation of the virus. Combining deletions in the E1 and
E3 regions of the viral genomes increases the maximum size of
the foreign that can be encapsulated to approx. 8.3 kb. In
addition, some sequences of the E4 region appear to be

20 dispensable for virus growth (adding another 1.8 kb to the
maximum encapsulation capacity). Also the E2A region can be
deleted from the vector, when the E2A gene product is
provided in trans in the encapsulation cell line, adding
another 1.6 kb. It is, however, unlikely that the maximum

25 capacity of foreign DNA can be significantly increased
further than 12 kb.

        We developed a new strategy for the generation and
production of helperfree-stocks of recombinant adenovirus
vectors that can accommodate up to 38 kb of foreign DNA. Only

30 two functional ITR sequences, and sequences that can function
as an encapsulation signal need to be part of the vector
genome. Such vectors are called minimal adenovectors. The
helper functions for the minimal adenovectors are provided in
trans by encapsulation defective-replication competent DNA

35 molecules that contain all the viral genes encoding the
required gene products, with the exception of those genes

that are present in the host-cell genome, or genes that
reside in the vector genome.
Ad D) The generation of a new adenovirus vector with the
means and methods in the prior art is possible. However, the
5   prior art means and methods are not very efficient in
generating adenovirus vector and moreover in the process of
generating said adenovirus vector many other vectors or even
replication competent adenovirus is produced necessitating a
thorough and elaborate evaluation of the generated viruses.
10   This is not a desired feature, specifically for clinical
settings the presence of replication competent adenovirus is
extremely undesired. In addition, specifically in settings
where many different adenovirus vectors need to be generated,
such as to produce expression libraries in adenovirus vectors
15   for instance for use in high throughput screenings, the
efficiency and the reliability of prior art adenovirus vector
production systems is as yet insufficient. Reliability of
adenovirus vector production is usually measured by means of
determining from a number of independent adenovirus vectors
20   produced, the number of independent adenovirus vectors
capable of functionally expressing a nucleic acid of interest
or an analogous method capable of determining an analogous
value for instance in case said vector is not intended to
express a nucleic acid of interest. Preferably at least 80%,
25   more preferably at least 90% and most preferably at least 95%
of the adenovirus vectors produced in a method of the
invention are functional vectors and when present in said
vector are capable of expressing said transgene and/or
nucleic acid of interest. Reliability of adenovirus vector
30   production is desired especially in applications where many
different adenovirus vectors need to be produced in a
relatively short time span. A reliable system for the
production of adenovirus vectors then can significantly
reduce the time and the costs involved. Preferably the
35   efficiency of adenovirus vector production for an average
vector is greater than 1 independent vector produced per 10^6

cells, more preferably the efficiency is more than 1
different vector produced 2 x 10^5 cells, most preferably the
efficiency is more than 1 different vector produced 5 x 10^4
cells.

        The applications of the disclosed inventions are
outlined below and are illustrated in the Examples.

<u>Use of the IG packaging constructs Diploid cells</u>
        The constructs, in particular pIG.E1A.E1B, will be
used to transfect diploid human cells, such as Human
Embryonic Retinoblasts (HER), Human Embryonic Kidney cells
(HEK), and Human Embryonic Lung cells (HEL). Transfected
cells will be selected for transformed phenotype (focus
formation) and tested for their ability to support
propagation of E1-deleted recombinant adenovirus, such as
IG.Ad.MLPI.IK. Such cell lines will be used for the
generation and (large-scale) production of E1-deleted
recombinant adenoviruses. Such cells, infected with
recombinant adenovirus are also intended to be used *in vivo*
as a local producer of recombinant adenovirus, e.g. for the
treatment of solid tumors. 911 cells are used for the
titration, generation and production of recombinant
adenovirus vectors (Fallaux *et al*, (1996) *Hum. Gene Ther.*
7:215-222).
        HER cells transfected with pIG.E1A.E1B has resulted
in 7 independent clones (called PER cells). These clones are
used for the production of E1 deleted (including non-
overlapping adenovirus vectors) or E1 defective recombinant
adenovirus vectors and provide the basis for introduction of
e.g. E2B or E2A constructs (e.g. ts125E2A, *see* below), E4
etc., that will allow propagation of adenovirus vectors that
have mutations in *e.g.* E2A or E4. In addition, diploid cells
of other species that are permissive for human adenovirus,
such as the cotton rat *(Sigmodon hispidus)* *(Pacini et al,*

(1984) *J. Infect. Dis.* 150:92-97), Syrian hamster (Morin *et al*, (1987) *Proc. Natl. Acad Sci. USA* 84:4626-4630) or chimpanzee (Levrero *et al*, (1991) *Gene* 101:195-202), will be immortalized with these constructs. Such cells, infected with

5   recombinant adenovirus, are also intended to be used *in vivo* for the local production of recombinant adenovirus, *e.g.* for the treatment of solid tumors.

Established cells

10   The constructs, in particular pIG.E1A.NEO, can be used to transfect established cells, *e.g.* A549 (human bronchial carcinoma), KB (oral carcinoma), MRC-5 (human diploid lung cell line) or GLC cell lines (small cell lung cancer) de Leij *et al*, (1985) *Cancer Res.* 45:6024-6033;

15   Postmus *et al*, (1988) *Eur. J. Clin. Oncol.* 24:753-763) and selected for NEO resistance. Individual colonies of resistant cells are isolated and tested for their capacity to support propagation of E1-deleted recombinant adenovirus, such as IG.Ad.MLPI.TK. When propagation of E1 deleted viruses on E1A

20   containing cells is possible, such cells can be used for the generation and production of E1-deleted recombinant adenovirus. They are also used for the propagation of E1A deleted/E1B retained recombinant adenovirus.

          Established cells can also be co-transfected with

25   pIG.E1A.E1B and pIG.NEO (or another NEO containing expression vector). Clones resistant to G418 are tested for their ability to support propagation of E1 deleted recombinant adenovirus, such as IG.Ad.MLPI.TK and used for the generation and production of E1 deleted recombinant adenovirus and will

30   be applied *in vivo* for local production of recombinant virus, as described for the diploid cells *(see above)*.

          All cell lines, including transformed diploid cell lines or NEO-resistant established lines, can be used as the basis for the generation of "next generation" packaging cell

35   lines, that support propagation of E1-defective recombinant

adenoviruses, that also carry deletions in other genes, such as E2A and E4. Moreover, they will provide the basis for the generation of minimal adenovirus vectors as disclosed herein.

5   <u>E2 expressing cell lines</u>

Packaging cells expressing E2A sequences are and will be used for the generation and (large scale) production of E2A-deleted recombinant adenovirus.

10   The newly generated human adenovirus packaging cell lines or cell lines derived from species permissive for human adenovirus (E2A or ts125E2A; E1A + E2A; E1A + E1B + E2A; E1A + E2A/ts 125; E1A + E1B + E2A/ts125) or non-permissive cell lines such as monkey cells (hrE2A or hr + ts125E2A; E1A +

15   hrE2A; E1A + E1B + hrE2A; E1A + hrE2A/ts125; E1A + E1B + hrE2A/ts 125) are and will be used for the generation and (large scale) production of E2A deleted recombinant adenovirus vectors. In addition, they will be applied *in vivo* for local production of recombinant virus, as described for

20   the diploid cells *(see above)*.

Novel adenovirus vectors

     The newly developed adenovirus vectors harboring an
El deletion of nt. 459-3510 will be used for gene transfer
purposes. These vectors may also be the basis for the
development of further deleted adenovirus vectors that are
mutated for e.g. E2A, E2B or E4. Such vectors will be
generated e.g. on the newly developed packaging cell lines
described above *(see 1-3)*.

Minimal adenovirus packaging system

     We disclose adenovirus packaging constructs (to be
used for the packaging of minimal adenovirus vectors) may
have the following characteristics:

a) the packaging construct replicates

b) the packaging construct cannot be packaged because the
   packaging signal is deleted.

c) the packaging construct contains an internal hairpin-
   forming sequence *(see* section "Experimental; suggested
   hairpin" *see* Fig. 15)

d) because of the internal hairpin structure, the packaging
   construct is duplicated, that is the DNA of the packaging
   construct becomes twice as long as it was before
   transfection into the packaging cell (in our sample it
   duplicates from 35 kb to 70 kb). This duplication also
   prevents packaging. Note that this duplicated DNA molecule
   has ITR's at both termini *(see e.g.* Fig. 13)

e) this duplicated packaging molecule is able to replicate
   like a "normal adenovirus" DNA molecule

f) the duplication of the genome is a prerequisite for the
   production of sufficient levels of adenovirus proteins,
   required to package the minimal adenovirus vector

g) the packaging construct has no overlapping sequences with
   the minimal vector or cellular sequences that may lead to
   generation of RCA by homologous recombination.

     This packaging system will be used to produce minimal
adenovirus vectors. The advantages of minimal adenovirus

vectors e.g. for gene therapy of vaccination purposes, are
well known (accommodation of up to 38 kb; gutting of all
potentially toxic and immunogenic adenovirus genes).

Adenovirus vectors containing mutations in essential
5 genes (including minimal adenovirus vectors) can also be
propagated using this system.

Use of intracellular E2-expressing vectors

Minimal adenovirus vectors are generated using the
10 helper functions provided in trans by packaging-deficient
replicating helper molecules. The adenovirus-derived ITR
sequences serve as origins of DNA replication in the presence
of at least the E2-gene products. When the E2 gene products
are expressed from genes in the vector genome (N.B. the
15 gene(s) must be driven by an E1-independent promoter), the
vector genome can replicate in the target cells. This will
allow a significantly increased number of template molecules
in the target cells, and, as a result an increased expression
of the genes of interest encoded by the vector. This is of
20 particular interest for approaches of gene therapy in cancer.

Applications of intracellular amplification of linear
DNA fragments

A similar approach could also be taken if amplification
25 of linear DNA fragments is desired. DNA fragments of known or
unknown sequence could be amplified in cells containing the
E2-gene products if at least one ITR sequence is located near
or at its terminus. There are no apparent constraints on the
size of the fragment. Even fragments much larger than the
30 adenovirus genome (36 kb) should be amplified using this
approach. It is thus possible to clone large fragments in
mammalian cells without either shuttling the fragment into
bacteria (such as E. coli) or use die polymerase chain
reaction (P.C.R.). At the end stage of a productive
35 adenovirus infection a single cell can contain over 100,000
copies of the viral genome. In the optimal situation, the

linear DNA fragments can be amplified to similar levels.
Thus, one should be able to extract more than 5 jig of DNA
fragment per 10 million cells (for a 35-kbp fragment). This
system can be used to express heterologous proteins
(equivalent to the Simian Virus 40-based COS-cell system) for
research or for therapeutic purposes. In addition, the system
can be used to identify genes in large fragments of DNA.
Random DNA fragments may be amplified (after addition of
ITRS) and expressed during intracellular amplification.
Election or, selection of those cells with the desired
phenotype can be used to enrich the fragment of interest and
to isolate the gene.

Gene correction vectors.

        Gene therapy procedures may be divided into two
different concepts, i.e., gene addition and gene replacement.
Gene addition aims at introducing a therapeutic nucleic acid
molecule into somatic cells of a patient, whereby expression
of said therapeutic nucleic acid molecule is often under the
control of a heterologous promoter and transcription
termination signal. For example, when a patient suffers from
an inherited disease, a functional copy of the defective
nucleic acid molecule responsible for the disease phenotype
is introduced into cells of the patient and, upon expression
of said therapeutic nucleic acid molecule, the disease
phenotype is corrected. Gene addition is, obviously, also
used to accomplish expression of otherwise not expressed
genes, such as, e.g., cytokine or suicide genes like HSV-TK
to treat tumors. The gene replacement procedure aims at
repairing at least one copy of a defective gene responsible
for a disease phenotype. This can be achieved by introducing
a functional version of a gene, or part thereof comprising
the mutant site of that gene, in such a way that homologous
recombination between said functional version and said
defective gene occurs. Consequently, the defective gene or
its mutant site is replaced by the functional version of that

gene or part thereof. In this way, no nucleic acid material that is foreign to the species of which the patient is a member is expressed in the treated cells but at least one allele of the mutant gene is repaired. For the majority of

5   the inherited diseases it is known that heterozygous carriers are not affected, or at least are affected to a lesser extent than a homozygous patient. Thus, gene replacement may be used for correction of inherited disorders. It is to be understood that this also includes the repair of defective tumor

10   suppressor genes.

        For gene therapy purposes, it is preferable to retain the E3 region. E3 containing vectors will be superior to their E3 deleted counterparts because they are able to prevent or reduce host cells responses such as CTL lysis of

15   adenovirus infected cells and cell lysis by TNF.

        It will be understood that it may not be necessary to retain the whole E3 region in the vectors according to the invention, as long as the part retained still has the function of reducing the response of the host against

20   infected cells. For example, expression of E3-14.7 kD alone may be sufficient to reduce early responses mediated by TNF (see Ginsberg, H.S. (1989) Proc. Natl. Acad Sci. USA 86:3823-3827. Ginsberg, H.S. (1991) Proc. Natl. Acad. Sci. USA 88:1651-1655). These vectors are useful for gene therapy of

25   inherited diseases such as cystic fibrosis, Duchenne molecular dystrophy, Hypercholesterolemia, blood clotting disorders (hemophilia) and the like. They also are useful in the therapy of acquired diseases, such as tumors, hepatitis, (auto) immune diseases, restenosis, rheumatoid and the like.

30   Advantages of gene replacement over gene addition include (1) expression regulation of the replacing gene is identical to the endogenous expression pattern, and (2) the procedure is safe, because no risk exists of insertion mutagenesis due to random integration.

35

A recombinant nucleic acid based system for the generation of adenovirus vectors.

In one aspect the invention provides a method for
5   generating an adenovirus vector comprising welding together
two nucleic acid molecules whereby said molecules comprise
partially overlapping sequences capable of combining with
each other allowing the generation of a physically linked
nucleic acid comprising at least two functional adenovirus
10   inverted terminal repeats, a functional encapsulation signal
and a nucleic acid of interest or functional parts,
derivatives and/or analogues thereof. Said nucleic acid
molecules together comprise at least a left ITR, a right ITR
and an adenovirus encapsulation signal or functional parts,
15   derivatives and/or analogues thereof. With overlapping
sequences are meant sequences that comprise sufficient
nucleic acid sequence similarity to allow homologous
recombination. Sequence similarity is preferably more than
80% and more preferably more than 95%. With overlapping
20   sequences are also meant complementary ends, of for instance
a restriction enzyme site, wherein said nucleic acids are
linked through hybridisation of said complementary ends. Said
welding together may be performed through any means capable
of physically linking two nucleic acid molecules. Preferably
25   said welding together is performed through linking
complementary ends resulting from restriction enzyme
digestion of said nucleic acid molecules. More preferably
said welding together is performed through homologous
recombination of overlapping sequences in said nucleic acid.
30   In one embodiment the invention provides a method for
generating an adenovirus vector comprising welding together
through homologous recombination two nucleic acid molecules
comprising partially overlapping sequences wherein said
overlapping sequences allow essentially only one homologous
35   recombination whichleads to the generation of a physically
linked nucleic acid comprising at least two functional

adenovirus inverted terminal repeats, a functional
encapsulation signal and a nucleic acid of interest or
functional parts, derivatives and/or analogues thereof. Very
important in this embodiment of the invention is that said

5   partially overlapping sequences allow essentially only
homologous recombination leading to the generation of a
functional adenovirus vector capable of being replicated and
packaged into adenovirus particles in the presence of the
required transacting functions. With essentially only one is

10   meant that said overlapping sequences in each nucleic acid
comprise essentially only one continuous sequence wherein
homologous recombination leading to the generation of a
functional adenovirus may occur. Within said continuous
sequence the actual number of homologous recombination events

15   may be higher than one. Non continuous overlapping sequences
are not desired because they reduce the reliability of said
method. Non continuous overlapping sequences are also not
desired because they reduce the overall efficiency of said
method, presumably due to the generation of undesired

20   homologous recombination products.

            A preferred embodiment of the invention provides a
method of the invention wherein both of said nucleic acid
molecules comprise only one adenovirus inverted terminal
repeat or a functional part, derivative and/or analogue

25   thereof.

            In one aspect of the invention one or both of said
two nucleic acid molecules have undergone modifications prior
to said welding together. Said modification may include the
welding together of different nucleic acid molecules leading

30   to the generation of one or both of said two nucleic acid
molecules. In a preferred embodiment said different nucleic
acids are welded together through homologous recombination
of partially overlapping sequences.

            In one aspect of the invention said welding together

35   is performed in a cell or a functional part, derivative
and/or analogue thereof. Preferably said cell is a mammalian

cell. In a preferred embodiment said nucleic acid molecules
are not capable of replicating in said mammalian cell prior
to said welding together. Said replication is undesired since
it reduces the reliability of the methods of the invention

5   presumably through providing additional targets for undesired
homologous recombination. Said replication is also not
desired because it reduces the efficiency of the methods of
the invention presumably because said replication competes
for substrate or adenovirus transacting functions with the

10   replication of said adenovirus vector.

In a preferred embodiment, one of said nucleic acid
molecules is relatively small and the other is relatively
large. This configuration is advantageous because it allows
easy manipulation of said relatively small nucleic acid

15   molecule allowing for example the generation of a large
number of small nucleic acid molecules comprising different
nucleic acid of interest for instance for the generation of
an adenovirus vector library. Said configuration is also
desired because it allows the production of a large batch of

20   quality tested large nucleic acid molecule. The amplification
of large nucleic acid molecules for instance in bacteria is
difficult in terms of obtaining sufficient amounts of said
large nucleic acid. The amplification of large nucleic acid
molecules for instance in bacteria is also difficult to

25   control because a small modification of said large nucleic
acid is not easily detected. Moreover, for reasons not quite
understood some large vectors are more stable in bacteria or
yeasts than others. Said configuration however, allows the
generation of a standard batch of a large nucleic acid

30   molecule which can be thoroughly tested, for instance through
generating a control adenovirus of which the efficiency and
the reliability of production is known, and determining said
parameters of a new batch of large nucleic acid molecule.
Once validated said batch may be used for the generation of a

35   large number of different adenovirus vectors through
combining said large molecule with a large number of

different small nucleic acid molecules. Said system therefore also allows for the selection and/or manipulation of vectors comprising a large nucleic acid molecule of the invention to allow a suitable yield of intact large nucleic acid.

5   In one embodiment of the invention at least one of said nucleic acid molecules comprises an adenovirus inverted terminal repeat which on one side is essentially free of other nucleic acid. An on one side essentially free adenovirus inverted terminal repeat is not essential for the

10 generation of an adenovirus vector with a method or a means of the invention. However, an on one side essentially free adenovirus inverted terminal repeat enhances the efficiency of adenovirus vector production as compared to not essentially free inverted terminal repeat. With essentially

15 free is meant that the outwardly directed end of said inverted terminal repeat is essentially free of additional nucleic acid bases. Some additional bases does not significantly affect the generation of adenovirus vectors particularly if said additional bases are not more than 50

20 bases, and preferably not more than 30 bases and more preferably not more than 10 bases. Preferably, both the left ITR and the right ITR are made essentially free of other nucleic acid on the outwardly directed side. Preferably, said adenovirus inverted terminal repeat is made essentially free

25 of other nucleic acid on one side through restriction enzyme digestion of a restriction enzyme site present near said adenovirus inverted terminal repeat. Preferably, said restriction enzyme site is not present anywhere else in nucleic acid destined to be part of the adenovirus vector, in

30 said nucleic acid molecule.

        In one aspect the invention provides a method of the invention for the generation of an adenovirus vector wherein the nucleic acids present in said cell do not comprise sequence overlap leading to the formation of replication

35 competent adenovirus. Other systems for the generation of adenovirus vectors do not sufficiently suppress the

generation of replication competent adenovirus upon the
generation and/or the propagation of adenovirus vectors. In
one aspect of the present invention the generation of
replication competent adenovirus is prevented through a
5   system that prevents potential homologous recombination
between for instance an E1-region and an adenovirus vector.
Preventing the generation of replication competent adenovirus
is very much desired when adenovirus vectors are to be used
in a clinical setting. Preventing the generation of
10   replication competent adenovirus increases the reliability of
adenovirus production. Preventing the generation of
replication competent adenovirus also increases the
efficiency of adenovirus vector production.

        One embodiment of the invention provides a method of
15   the invention wherein the chromosomal nucleic acid in said
cell comprises at least a functional part of an adenovirus
E1-region, or a functional derivative and/or analogue
thereof. Preferably said cell is a PER.C6 cell (ECACC deposit
number 96022940) or a functional derivative and/or analogue
20   thereof.

        In another embodiment said nucleic acid in said cell
further comprises a nucleic acid encoding an adenovirus E2-
region and/or an adenovirus E4-region protein.

        In another embodiment the invention provides a method
25   or means for the generation of an adenovirus vector, wherein
at least one of said nucleic acid molecules is linear. A
linear molecule is not essential for adenovirus vector
production however the efficiency of adenovirus vector
production is increased compared to when circular or
30   supercoiled molecules are used.

        In one embodiment the invention provides a method
according to the invention, wherein at least one of said
molecules comprises adenovirus capsid proteins encoding
nucleic acid derived from at least two different adenovirus
35   serotypes. This embodiment of the invention is useful for the
generation of for instance an adenovirus particle with a

chimeric capsid comprising proteins from at least two
different adenovirus serotypes. One advantageous feature of a
chimeric capsid is the capacity to alter the tissue tropism
of an adenovirus vector. The capsid of an adenovirus particle
5   is among others a major determinant of whether a particle is
capable of entering a certain cell type (tissue tropism) and
by altering the capsid the tissue tropism of an adenovirus
vector can be altered and designed to meet specific needs.
Preferably said capsid comprises at least a tissue tropism
10 determining part of a fiber protein of a subgroup B type
adenovirus such as adenovirus 16 and at least one other
capsid protein derived from a subgroup C type adenovirus such
as adenovirus 5. Preferably said nucleic acid molecule
comprising said adenovirus capsid protein encoding nucleic
15 acid is a large nucleic acid molecule, thus enabling the easy
generation of a library of adenovirus vectors packaged into
said chimeric capsid.
        In another embodiment the invention provides a method
of the invention wherein said welding together of said
20 nucleic acid molecules leads to the generation of a
physically linked nucleic acid comprising at least two
functional adenovirus inverted terminal repeats, a functional
encapsulation signal, a nucleic acid encoding at least one
adenovirus E1-region protein, at least one adenovirus E2-
25 region encoded protein and/or at least one adenovirus E4-
region encoded protein and a nucleic acid of interest or
functional parts, derivatives and/or analogues thereof and
wherein at least one of said E1-region encoded proteins is
under transcriptional control of a conditionally active
30 promoter. With a conditionally active promoter is meant a
promoter active in certain cell types and inactive in other
cell types. The method of this embodiment is particularly
useful for generation of a molecule capable of replicating in
a cell provided that the conditionally active promoter is
35 active in said cell. Such a molecule is useful for instance
in vaccinations where very high expression of a transgene is

required specifically in antigen presenting cells. When a
vector of this embodiment is further provided with the
capacity to express adenovirus capsid proteins said vector
capable of replicating is also capable of being packaged in a
5   cell provided that the conditionally active promoter is
active in said cell. Thus forming a conditional replication
incompetent adenovirus vector.

In another embodiment the invention provides a method
of the invention wherein said physically linked nucleic acid
10   comprises no other functional adenovirus nucleic acid than
two inverted terminal repeats and a functional packaging
signal or functional parts, derivatives and/or analogues
thereof and wherein said physically linked nucleic acid is
generated through welding together two nucleic acid molecules
15   said molecules comprising partially overlapping sequences
capable of combining with each other allowing the generation
said physically linked nucleic acid. Said physically linked
nucleic acid preferably further comprises a nucleic acid of
interest. This setting is favorable is it allows the rapid
20   generation of minimal adenovirus vectors through combining
different relatively small nucleic acid molecules comprising
different nucleic acids of interest with one tested and
validated large nucleic acid molecule.

25   The invention further provides a method for generating
an adenovirus vector with a deletion of the E2A gene
comprising providing a cell with said nucleic acid molecules
and growing said cell wherein a first nucleic acid comprises
an adenovirus inverted terminal repeat and an encapsulation
30   signal or functional parts, derivatives and/or analogues
thereof, and a partially overlapping sequence allowing it to
be welded together with a second nucleic acid molecule
comprising an adenovirus inverted terminal repeat or a
functional part, derivative and/or analogue thereof, a
35   deletion of at least part of the E2A gene and a partially
overlapping sequence, wherein said cell is capable of

expressing functional E2A, preferably a temperature sensitive
E2A. Both or either one of said nucleic acids may further
comprise a nucleic acid of interest operatively linked to a
transcription unit like a promoter and poly-adenylation
5   signal or functional parts, derivatives and or analogues
thereof. Welding together of said partially overlapping
sequences may be achieved through any means provided capable
of faithfully joining two strands of nucleic acid. Preferably
said partially overlapping sequences are welded together
10   through one homologous recombination. In a preferred
embodiment said second nucleic acid is deleted of at least
all E2A sequences that are present in said cell thus
preventing homologous recombination that abolishes said
deletion of E2A in said second nucleic acid or derivatives
15   thereof resulting from said welding together.

        The invention further provides a method for generating
replication competent adenoviruses with a deletion in the E3
region comprising providing a cell with nucleic acid
molecules and growing said cell wherein a first nucleic acid
20   comprises an adenovirus inverted terminal repeat and
encapsulation signal or functional parts, derivatives and/or
analogues thereof, a conditionally functional E1 region, and
a partially overlapping sequence allowing it to recombine
with a second nucleic acid molecule comprising an adenovirus
25   inverted terminal repeat or a functional part, derivative
and/or analogue thereof, a deletion in the E3 region and a
partially overlapping sequence. Preferably said second
nucleic acid contains a nucleic acid of interest in the E3
region. More preferably said nucleic acid of interest is
30   operatively linked to the E3 promoter. Said nucleic acid of
interest may be a suicide gene, a cytokine or a marker gene.
Said second nucleic acid molecule may be generated in said
cell by homologous recombination of two partially overlapping
smaller nucleic acid molecules one of which comprises said
35   deletion in the E3 region with or without a nucleic acid of
interest and only one of said smaller nucleic acid molecules

contains an adenoviral inverted terminal repeat preferably positioned on the end of one of said smaller nucleic acid molecules opposite of the partially overlapping sequence. The invention further provides a method to generate

5   adenoviral vectors with a modification in at least one of the late genes comprising providing a cell with nucleic acid molecules and growing said cell wherein a first nucleic acid molecule comprises an adenovirus inverted terminal repeat and an encapsulation signal or functional parts, derivatives

10   and/or analogues thereof, and a partially overlapping sequence allowing it to recombine with a second nucleic acid molecule comprising an adenovirus inverted terminal repeat or a functional part, derivative and/or analogue thereof, a modification in at least one of the late genes and a

15   partially overlapping sequence. Said modification in at least one of the late genes may comprise a modification in one of the capsid proteins, preferably penton or hexon or fiber or more preferably in more than one of the capsid proteins, more preferably in penton, hexon and fiber. Said modification may

20   be a change in the nucleotide sequence resulting from mutagenesis, deletion, insertion or combinations thereof leading to a functional change of said adenoviral vector in for example immunogenicity, infectivity or stability. Preferably said modification is a modification of said capsid

25   genes generated by exchange of complete or part(s) of equivalent capsid genes from one or more different human or animal adenovirus serotypes, leading to a functional change of said adenoviral vector in for example immunogenicity, infectivity or stability. Said second nucleic acid molecule

30   may be generated in said cell by homologous recombination of two partially overlapping smaller nucleic acid molecules at least one of which comprises said modification in one or more capsid genes whereby only one of said smaller nucleic acid molecules contains an adenoviral inverted terminal repeat or

35   a functional part, derivative and/or analogue thereof, preferably positioned at the end of one of said smaller

41

nucleic acid molecules opposite of said partially overlapping
sequence.

The invention further provides a recombinant nucleic
acid deposited under No. P97082122 at the ECACC.

5   The invention further provides a recombinant nucleic
acid deposited under No. P97082119 at the ECACC.

The invention further provides a recombinant nucleic
acid deposited under No. P97082117 at the ECACC.

The invention further provides a recombinant nucleic
10   acid deposited under No. P97082114 at the ECACC.

The invention further provides a recombinant nucleic
acid deposited under No. P97082120 at the ECACC.

The invention further provides a recombinant nucleic
acid deposited under No. P97082121 at the ECACC.

15   The invention further provides a recombinant nucleic
acid deposited under No. P97082116 at the ECACC.

The invention further provides a recombinant nucleic
acid deposited under No. P97082115 at the ECACC.

The invention further provides a recombinant nucleic
20   acid deposited under No. P97082118 at the ECACC.

The invention further provides a recombinant nucleic
acid pWE/Ad.AflII-EcoRI.

The invention further provides a recombinant nucleic
acid comprising:

25   adenovirus derived nucleotides 1-454 and adenovirus
nucleotides 3511-6095 shown in Figures 21 and 22.

The invention further provides a recombinant nucleic
acid pAd5/CLIP.

The invention further provides a recombinant nucleic
30   acid pAd5/L420-HSA.

The invention further provides a recombinant nucleic
acid pBS.Eco-Eco/ad5ΔHIIIΔgp19KΔXbaI.

The invention further provides a recombinant nucleic
acid according to the invention, wherein said nucleic acid
35   further comprises a transgene.

The invention further provides a recombinant nucleic
acid according to the invention, wherein said transgene is
operatively linked to an E3 promoter.

The invention further provides a recombinant nucleic
5   acid according to the invention, wherein said transgene
comprises a suicide gene, a cytokine gene, or a reporter
gene.

The invention further provides a recombinant nucleic
acid according to the invention, wherein said suicide gene is
10 an HSV-TK gene.

The invention further provides a recombinant nucleic
acid according to the invention, wherein said transgene
comprises a coding sequence selected from the group
consisting of hIL-1α, rat IL-3, and human IL-3.

15   The invention further provides a recombinant nucleic
acid according to the invention, wherein said transgene
comprises a coding sequence from a luciferase gene or a LacZ
gene.

The invention further provides a recombinant nucleic
20 acid according to the invention, wherein said transgene
comprises a coding sequence from a human ceNOS gene.

The invention further provides a recombinant nucleic
acid of the invention comprising a deletion in an E3 region
of a recombinant nucleic acid.

25   The invention further provides a recombinant nucleic
acid of the invention comprising a deletion in a gp19K region
of a recombinant nucleic acid.

The invention further provides a recombinant nucleic
acid comprising a nucleotide sequence based on or derived
30 from an adenovirus, wherein said nucleotide sequence
comprises a functional encapsulating signal and two
functional Inverted Terminal Repeats or functional fragments
or derivatives thereof, and wherein said recombinant nucleic
acid has no functional adenovirus genes and no overlapping
35 sequences which allow for homologous recombination leading to
replication competent virus in a cell into which said

recombinant nucleic acid is transferred. Preferably, said
recombinant nucleic acid, further comprising a heterologous
nucleotide sequence.

The invention further provides a recombinant nucleic
5   acid pMV/L420-H.

The invention further provides a recombinant nucleic
acid pMV/CMV-LacZ.

The invention further provides a recombinant nucleic
acid comprising:

10   a nucleotide sequence based on or derived from an
adenovirus, wherein said nucleotide sequence comprises
sufficient adenovirus sequences necessary for replication and
capsid gene expression, wherein said nucleotide sequence
comprises a deletion of at least the E1 region and

15   encapsulating signal of said adenovirus, and wherein said
nucleotide sequence comprises no sequences which allow for
homologous recombination leading to replication competent
virus in a cell into which said recombinant nucleic acid is
transferred.

20   The invention further provides a recombinant nucleic
acid pWE/Ad.Δ5'.

The invention further provides a recombinant nucleic
acid comprising:

a nucleotide sequence based on or derived from an
25   adenovirus, wherein said nucleotide sequence comprises
sufficient adenovirus sequences necessary for replication and
capsid gene expression, and a complementary sequence to an
upstream part of the same strand of said nucleic acid,
wherein said complementary sequence can base-pair with said

30   upstream part so that it functions as a start-site for a
nucleic acid polymerase, wherein said nucleotide sequence
comprises a deletion of one Inverted Terminal Repeat, the E1
region and the encapsulating signal of said adenovirus, and
wherein said nucleic acid has no overlapping sequence which

35   allow for homologous recombination leading to replication

competent virus in a cell into which said nucleic acid is transferred. Preferably said molecule is pWE/AAV.Δ5'.

The invention further provides a recombinant nucleic acid comprising:

5   a nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises a sequence for adenovirus-independent replication, and sufficient adenoviral sequences necessary for replication, wherein said nucleotide sequence comprises at least a

10   deletion of the E1 region and encapsulating signal of said adenovirus, and wherein said nucleic acid has no overlapping sequences which allow for homologous recombination leading to replication competent virus in a cell into which said nucleic acid is transferred. Preferably, said nucleotide sequence

15   further comprises a deletion of at least one of the Inverted Terminal Repeats of said adenovirus. Preferably, said sequence for adenovirus-independent replication comprises an SV40 origin of replication.

The invention further provides a recombinant nucleic

20   acid pWE/Ad-H.

The invention further provides an adapter plasmid comprising:

    a nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises in

25   operable configuration at least one functional Inverted Terminal Repeat, one functional encapsulating signal and adenoviral sequences which allow for homologous recombination and the generation of a replication-defective, recombinant adenovirus genome, and wherein said adapter plasmid has no

30   sequences which allow for homologous recombination leading to replication competent virus in a cell into which said adapter plasmid is transferred. Preferably said adapter plasmid comprises no E1 region sequences. Preferably said adapter plasmid further comprises a multiple cloning site. Also

35   preferred is an adapter plasmid according to the invention,

further comprising a nucleic acid inserted into said multiple
cloning site.

In another embodiment the invention provides a method
for the generation of recombinant adenovirus having an E1
5   deletion and a gp19K deletion, comprising the step of:
growing a cell comprising adenovirus complementing
sequences transfected with

i) an adapter plasmid comprising a first nucleotide
sequence based on or derived from an adenovirus, wherein said
10   nucleotide sequence comprises in operable configuration one
functional Inverted Terminal Repeat, one functional
encapsulating signal and adenoviral sequences which allow for
homologous recombination leading to the generation of a
replication-defective, recombinant adenovirus genome in a
15   cell into which said adapter plasmid is transferred and
having no E1 region sequences, and

ii) a recombinant nucleic acid comprising at least
one second nucleotide sequence based on or derived from an
adenovirus, wherein said at least one second nucleotide
20   sequence comprises one Inverted Terminal Repeat and
sufficient adenovirus sequences for replication and a partial
overlap with said adapter plasmid, wherein said at least one
second nucleotide sequence comprises a deletion of at least
the E1 region, encapsulating signal and gp19K sequences;.
25   wherein said complementing sequences, said first nucleotide
sequence and said at least one second nucleotide sequence
have no overlapping sequences which allow for homologous
recombination leading to replication competent virus,
under conditions whereby recombinant adenovirus having an E1
30   deletion and a gp19K deletion is generated. Preferably, said
adapter plasmid further comprises a first heterologous
nucleotide sequence inserted into said E1 region deletion and
said recombinant nucleic acid further comprises a second
heterologous nucleotide sequence inserted into said gp19K
35   region.

In another embodiment the invention provides a method
for the generation of recombinant adenovirus, comprising the
step of:
    growing a cell comprising adenovirus complementing
5   sequences transfected with
        i) a first recombinant nucleic acid comprising a
first nucleotide sequence based on or derived from an
adenovirus, wherein said first nucleotide sequence comprises
a functional encapsulating signal and two functional Inverted
10   Terminal Repeats or functional fragments or derivatives
thereof, and wherein said first recombinant nucleic acid has
no functional adenovirus genes and
        ii) a second recombinant nucleic acid comprising a
second nucleotide sequence based on or derived from an
15   adenovirus, wherein said second nucleotide sequence comprises
sufficient adenovirus sequences for replication, wherein said
second nucleotide sequence comprises a deletion of at least
the E1 region and encapsulating signal of said adenovirus;
        wherein, said complementing sequences, said first
20   nucleotide sequence and said second nucleotide sequence have
no overlapping sequences which allow for homologous
recombination leading to replication competent virus,
        under conditions whereby recombinant adenovirus is
generated.
25   In another embodiment the invention provides a method
for the generation of recombinant adenovirus, comprising the
steps of:
        growing a cell comprising adenovirus complementing
sequences transfected with
30   i) a first recombinant nucleic acid comprising a
first nucleotide sequence based on or derived from an
adenovirus, wherein said first nucleotide sequence comprises
a functional encapsulating signal and two functional Inverted
Terminal Repeats or functional fragments or derivatives
35   thereof, and wherein said first recombinant nucleic acid has
no functional adenovirus genes and

ii) a second recombinant nucleic acid comprising a
second nucleotide sequence based on or derived from an
adenovirus, wherein said second nucleotide sequence comprises
a sequence for adenovirus-independent replication, and
5 sufficient adenoviral sequences necessary for replication,
wherein said second nucleotide sequence comprises at least a
deletion of the E1 region and encapsulating signal of said
adenovirus;

wherein, said complementing sequences, said first
10 nucleotide sequence and said second nucleotide sequence have
no overlapping sequences which allow for homologous
recombination leading to replication competent virus,

under conditions whereby recombinant adenovirus is
generated. Preferably said cell comprises at least one
15 nucleic acid molecule whereby said cell expresses SV40 Large
T antigen proteins or functional fragments thereof. More
preferably said second recombinant nucleic acid molecule is
replicated.

In one embodiment the invention provides a
20 replication defective adenovirus comprising:

a genome based on or derived from an adenovirus,
wherein said genome comprises at least a functional
encapsulating signal and two functional Inverted Terminal
Repeats or functional fragments or derivatives thereof and
25 wherein said genome comprises no functional adenovirus genes
and has no overlapping sequences which allow for homologous
recombination leading to replication competent virus in a
cell into which said replication defective adenovirus is
transferred. Preferably said replication defective adenovirus
30 further comprises one or more expression cassettes.
Preferably, said expression cassette comprises a gene
functionally linked to transcription regulatory sequences.

In one embodiment said replication defective
adenovirus further comprises one or more non-adenoviral
35 nucleic acid sequences. Preferably said one or more non-

adenoviral nucleic acid sequences are inserted in the E1 region or in the E3 region gp19K gene.

In another aspect the invention provides a non-human cell comprising a genome of a replication defective

5   adenovirus according to the invention. Preferably said cell is a mammalian cell.

In another aspect the invention provides a method for transducing a cell, comprising the step of:

contacting said cell with a replication defective

10   adenovirus according to the invention under conditions whereby said cell is transduced.

In another aspect the invention provides a non-human cell produced according to a method of the invention, preferably said cell is a mammalian cell.

15   In one embodiment the invention provides a method for generating recombinant adenovirus comprising the step of:

growing a cell comprising adenovirus complementing sequences and

i) a first recombinant nucleic acid comprising a

20   first nucleotide sequence based on or derived from an adenovirus, wherein said first nucleotide sequence comprises a functional encapsulating signal and two functional Inverted Terminal Repeats or functional fragments or derivatives thereof, and wherein said first recombinant nucleic acid has

25   no functional adenovirus genes and,

ii) a second recombinant nucleic acid comprising a second nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises at least all adenovirus sequences, or functional fragments or

30   derivatives thereof necessary for replication and capsid gene expression, and a complementary sequence to an upstream part of the same strand of said nucleic acid, wherein said complementary sequence can base-pair with said upstream part so that it functions as a start-site for a nucleic acid

35   polymerase, wherein said second nucleotide sequence comprises

a deletion of one Inverted Terminal Repeat, the E1 region and
the encapsulating signal of said adenovirus;

    wherein, said complementing sequences, said first
nucleotide sequence and said second nucleotide sequence have
5 no overlapping sequences which allow for homologous
recombination leading to replication competent virus,

    under conditions whereby recombinant adenovirus is
generated.

    In another aspect the invention provides a cell
10 comprising a recombinant nucleic acid and/or an adapter
plasmid according to the invention.

    In yet a further aspect the invention provides a
method for the replacement of a defective gene in a host cell
genome comprising the step of:

15   growing said host cell with a recombinant nucleic
acid molecule derived from a replication defective adenovirus
comprising a functional version or part thereof of said
defective gene under conditions whereby at least one allele
of said defective gene in said host cell genome is replaced.

20   In one embodiment the invention provides a method for
transducing a cell according to the invention, wherein said
replication defective adenovirus expresses no adenoviral
genes. Preferably said defective gene is a defective tumor
suppressor gene.

25   The invention further provides an isolated cell
comprising a genome of a replication-defective adenovirus
according to the invention. Preferably said cell is a human
cell.

    The invention further provides a recombinant nucleic
30 acid according to the invention, wherein said deletion in the
E3 region is replaced with a transgene.

    The invention further provides a recombinant nucleic
acid according to the invention, wherein said deletion in the
gp19K region is replaced with a transgene.

35   The invention further provides a method according to
the invention, wherein said at least one second nucleotide

sequence comprises a first and second molecule wherein said
first molecule has said partial overlap with said adapter
plasmid at the 3' end, and said second molecule comprises
said Inverted Terminal Repeat and region including deletion

5   of said gp19K sequences.

         The invention further provides a replication-
defective adenovirus comprising:
a genome based on or derived from an adenovirus, wherein said
genome comprises a first deletion. in and E1 region and a

10   second deletion in a gp19K region. Preferably transcription
of said transgene is directed by an E3 promoter.

         The invention further provides an isolated cell
comprising:
a recombinant nucleic acid and/or an adapter plasmid

15   according to the invention. Preferably said cell is a human
cell.

         The following examples are presented as

20   illustrations, not limitations.

51

EXAMPLES

Example I
Generation of cell lines able to transcomplement E1
defective recombinant adenovirus vectors

5

1. 911 cell line

A cell line that harbors E1 sequences of adenovirus
type 5, able to trans-complement E1 deleted recombinant
adenovirus has been generated (Fallaux et al, (1996) *Hum.*

10 *Gene Ther.* 7:215-222). This cell line was obtained by
transfection of human diploid human embryonic retinoblasts
(HER) with pAd5XhoIC, that contains nt. 80-5788 of Ad 5; one
of the resulting transformants was designated 911. This cell
line has been shown to be useful in the propagation of E1

15 defective recombinant adenovirus. It was found to be superior
to the 293 cells. Unlike 293 cells, 911 cells lack a fully
transformed phenotype, which most likely is the cause of
performing better as adenovirus packaging line:

        plaque assays can be performed faster (4-5 days

20   instead of 8-14 days on 293) monolayers of 911 cells
        survive better under agar overlay as required for
        plaque assays higher amplification of E1-deleted
        vectors.

        In addition, unlike 293 cells that were transfected

25 with sheared adenoviral DNA, 911 cells were transfected using
a defined construct. Transfection efficiencies of 911 cells
are comparable to those of 293.

New packaging constructs

30 Source of adenovirus sequences

        Adenovirus sequences are derived either from
pAd5.SalB. containing nt. 80-9460 of human adenovirus type 5
(Bernards et al, (1983) *Virology* 127:45-53) or from wild-type
Ad5 DNA. PAd5.SalB was digested with *Sal*I *and Xho*I and the

35 large fragment was religated and this new clone was named

pAd5.X/S. The pTN construct (constructed by Dr. R. Vogels, IntroGene, The Netherlands) was used as a source for the human PGK promoter and the NEO gene.

5   Human PGK Promoter and NEO[R] gene

Transcription of E1A sequences in the new packaging constructs is driven by the human PGK promoter (Michelson et al, (1983) *Proc. Natl. Acad. Sci.* USA 80:472-476); Singer-Sam et al, (1984) *Gene* 32:409-417), derived from plasmid pTN

10   (gift of R. Vogels), which uses pUC 119 (Vieira *et al*, (1987) pp. 3-11: *Methods in Enzymology*, Acad. Press Inc.) as a backbone. This plasmid was also used as a source for the NEO gene fused to the Hepatitis B Virus (HBV) poly-adenylation signal.

15

Fusion of PGK promoter to E1 genes (Fig. 1)

In order to replace the E1 sequences of Ad5 (ITR, origin of replication and packaging signal) by heterologous sequences we have amplified E1 sequences (nt.459 to nt.960)

20   of Ad5 by PCR, using primers Ea1 and Ea2 *(see* Table 1). The resulting PCR product was digested with ClaI and ligated into Bluescript (Stratagene), predigested with *ClaI* and *EcoRV* resulting in construct pBS.PCRI.

Vector pTN was digested with restriction enzymes

25   *EcoRI* (partially) and *ScaI*, and the DNA fragment containing the PGK promoter sequences was ligated into PBS.PCRI digested with *ScaI* and *EcoRI*. The resulting construct PBS.PGK.PCRI contains the human PGK promoter operatively linked to Ad5 E1 sequences from nt.459 to nt.916.

<u>Construction of pIG.E1A.E1B (Fig. 2)</u>

pIG.E1A.E1B.X contains the E1A and E1 B coding
sequences under the direction of the PGK promoter. As Ad5
sequences from nt.459 to nt.5788 are present in this
5   construct, also pIX protein of adenovirus is encoded by this
plasmid. pIG.E1A.E1B.X was made by replacing the *ScaI-BspEI*
fragment of pAT-X/S by the corresponding fragment from
PBS.PGK.PCRI (containing the PGK promoter linked to E1A
sequences).
10

<u>Construction of pIG.E1A.NEO (Fig. 3)</u>

In order to introduce the complete E1B promoter and
to fuse this promoter in such a way that the AUG codon of E1B
21 kD exactly functions as the AUG codon of NEO^R, the EIB
15   promoter was amplified using primers Ea3 and Ep2, where
primer Ep2 introduces a *NcoI* site in the PCR fragment. The
resulting PCR fragment, named PCRII, was digested with *HpaI*
and NcoI and ligated into pAT-X/S, which was predigested with
HpaI and with *NcoI*. The resulting plasmid was designated pAT-
20   X/S-PCR2. The *NcoI-StuI* fragment of pTN, containing the NEO
gene and part of the Hepatitis B Virus (HBV) poly-adenylation
signal, was cloned into pAT-X/SPCR2 which had been digested
with *NcoI and NruI)*. The resulting construct was
pAT.PCR2.NEO. The poly-adenylation signal was completed by
25   replacing the *ScaI-SalI* fragment of pAT.PCR2.NEO with the
corresponding fragment of pTN, resulting in
pAT.PCR2.NEO.p(A). The *ScaI-XbaI* of pAT.PCR2.NEO.p(A) was
replaced with the corresponding fragment of pIG.E1A.E1B-X,
containing the PGK promoter linked to E1A genes. The
30   resulting construct was named pIG.E1A.NEO, and thus contains
Ad5 E1 sequences (nt.459 to nt.1713) under the control of the
human PGK promoter.

Construction of pIG.E1A.E1B (Fig. 4)

pIG.E1A.E1B contains nt.459 to nt.3510 of Ad5, that encode the E1A and E1B proteins. The E1B sequences are terminated at the splice acceptor at nt.3511. No pIX
5   sequences are present in this construct.

pIG.E1A.E1B was made as follows: The sequences encoding the N-terminal amino acids of E1B 55 kD were amplified using primers Eb1 and Eb2 which introduces a XhoI site. The resulting PCR fragment was digested with BglII and
10   cloned into BglII/NruI of pAT-X/S, thereby obtaining pAT-PCR3. The HBV poly (A) sequences of pIG.E1A.NEO were introduced downstream of the E1B sequences of pAT-PCR3 by exchange of the Xba-SalI fragment of pIG.E1A.NEO and the XbaI XhoI fragment of pAT.PCR3.

15

Construction of pIG.NEO (Fig. 5)

This construct is of use when established cells are transfected with EIA.E1B constructs and NEO selection is required. Because NEO expression is directed by the E1B
20   promoter, NEO resistant cells are expected to co-express. E1A, which also is advantageous for maintaining high levels of expression of E1A during long-term culture of the cells. pIG.NEO was generated by cloning the HpaI-ScaI fragment of pIG.E1A.NEO, containing the NEO gene under the control of the
25   Ad5 E1B promoter, into pBS digested with EcoRV and ScaI.

Testing of constructs

The integrity of the constructs pIG.E1A.NEO, pIG.E1A.E1B.X and pIG.E1A.E1B was assessed by restriction
30   enzyme mapping; furthermore, parts of the constructs that were obtained by PCR analysis were confirmed by sequence analysis. No changes in the nucleotide sequence were found.

The constructs were transfected into primary BRK (Baby Rat Kidney) cells and tested for their ability to
35   immortalize (pIG.E1A.NEO) or fully transform (pAd5.XhoIC,

pIG.E1A.E1B.X and pIG.E1A.E1B) these cells. Kidneys of 6-day old WAG-Rij rats were isolated, homogenized and trypsinized. Subconfluent dishes (diameter 5 cm) of the BRK cell cultures were transfected with 1 or 5 µg of pIG.NEO, pIG.E1A.NEO,

5 pIG.E1A.E1B, pIG/E1A.E1B.X, pAd5XhiIC, or with pIG.E1A.NEO together with PDC26 (Elsen et al, (1983) Virology 128:377-390), carrying the Ad5.E1B gene under control of the SV40 early promoter. Three weeks post-transfection, when foci were visible, the dishes were fixed, Giemsa stained and the foci

10 counted.

An overview of the generated adenovirus packaging constructs, and their ability to transform BRK, is presented in Fig. 6. The results indicate that the constructs pIG.E1A.E1B and pIG.E1A.E1B.X are able to transform BRK cells

15 in a dose-dependent manner. The efficiency of transformation is similar for both constructs and is comparable to what was found with the construct that was used to make 911 cells, namely pAd5.XhoIC.

As expected, pIG.E1A.NEO was hardly able to

20 immortalize BRK. However, co-transfection of an E1B expression construct (PDC26) did result in a significant increase of the number of transformants (18 versus 1), indicating that the E1A encoded by pIG.E1A.NEO is functional. We conclude therefore, that the newly generated packaging

25 constructs are suitable for the generation of new adenovirus packaging lines.

Generation of cell lines with new packaging constructs cell lines and cell culture

30 Human A549 bronchial carcinoma cells (Shapiro et al, (1978) Biochem. Biophys. Acta 530:197-207), human embryonic retinoblasts (HER), Ad5-E1-transformed human embryonic kidney (HEK) cells (293; Graham et al, (1977) J. Gen. Virol. 36:59-72) and Ad5-transformed HER cells (911; Fallaux et al,

35 (1996). Hum. Gene Ther. 7:215-222) and PER cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with

10% Fetal Calf Serum (FCS) and antibiotics in a 5% CO2
atmosphere at 37°C. Cell culture media, reagents and sera
were purchased from Gibco Laboratories (Grand Island, NY).
Culture plastics were purchased from Greiner (Nürtingen,
5   Germany) and Corning (Cooring, NY).

Viruses and virus techniques
         The construction of recombinant adenoviral vectors
IG.Ad.MLP.nls.lacZ, IG.Ad.NILP.luc, IG.Ad.MLP.TK and
10   IG.Ad.CW.TK is described in detail in patent application EP
95202213. The recombinant adenoviral vector
IG.Ad.MIP.nls.lacZ contains the E. coli lacZ gene, encoding
β-galactosidase, under control of the Ad2 major late promoter
(MLP), IG.Ad.MLP.luc contains the firefly luciferase gene
15   drive by the Ad2 MLP, and adenoviral vectors IG.Ad.NLP.TK and
IG.Ad.CMV.TK contain the Herpes Simplex Virus thymidine
kinase (TK) gene under the control of the Ad2 MLP and the
Cytomegalovirus (CMV) enhancer/promoter, respectively.

20   Transfections
         All transfections were performed by calcium-phosphate
precipitation DNA (Graham et al, (1973) Virology 52:456-467)
with the GIBCO Calcium Phosphate Transfection System (GEBCO
BRL Life Technologies, Inc., Gaithersburg, USA), according to
25   the manufacturer's protocol.

Western blotting
         Subconfluent cultures of exponentially growing 293,
911 and Ad5-E1-transformed A549 and PER cells were washed
30   with PBS and scraped in Fos-RIPA buffer (10 mM Tris (pH 7,5),
150 mM NaCl, 1% NP40,01% sodium dodecyl sulfate (SDS), 1%
NA-DOC, 0.5 mm phenyl methyl sulfonyl fluoride (PMSF), 0.5 mM
trypsin inhibitor, 50 mM NaF and 1 mM sodium vanadate). After
10 min. at room temperature, lysates were cleared by
35   centrifugation. Protein concentrations were measured with the
BioRad protein assay kit and 25 μg total cellular protein was

loaded on a 12.5% SDS-PAA gel. After electrophoresis,
proteins were transferred to nitrocellulose (1h at 300 mA).
Prestained standards (Sigma, USA) were run in parallel.
Filters were blocked with 1% bovine serum albumin (BSA) in

5　TBST (10 mM Tris, pH 8.15 mM NaCl, and 0.05% Tween-20) for
1 hour. First antibodies were the mouse monoclonal anti-Ad5-
E1B-55-kDA antibody A1C6 (Zantema et al, unpublished), the
rat monoclonal anti-Ad5-EIB-221-kDa antibody C1G11 (Zantema
et al, (1985) Virology 142:44-58). The second antibody was a

10　horseradish peroxidase-labeled goat anti-mouse antibody
(Promega). Signals were visualized by enhanced
chemoluminescence (Amersham Corp. UK).

Southern blot analysis

15　　　　　High molecular weight DNA was isolated and 10 μg was
digested to completion and fractionated on a 0.7% agarose
gel. Southern blot transfer to Hybond N+ (Amersham, UK) was
performed with a 0.4 M NAOH, 0.6 M NaCl transfer solution
(Church and Gilbert, 1984). Hybridization was performed with

20　a 2463-nt SspI-HindIII fragment from pAd5.SalB (Bernards et
al, (1983) Virology 127:45-53). This fragment consists of Ad5
bp. 342-2805. The fragment was radiolabeled with a, α^{-32P}=dCTP
with the use of random hexanucleotide primers and Klenow DNA
polymerase. The southern blots were exposed to a Kodak XAR-5

25　film at -80°C and to a Phosphor-Imager screen which was
analyzed by B&L systems Molecular Dynamics Software.

A549

　　　　　Ad5-E1-transformed A549 human bronchial carcinoma
30　cell lines were generated by transfection with pIG.E1A.NEO
and selection for G418 resistance. Thirty-one G418 resistant
clones were established. Co-transfection of pIG.E1A.E1B with
pIG.NEO yielded seven G418 resistant cell lines.

PER

Ad5-E1-transformed human embryonic retina (HER) cells
were generated by transfection of primary HER cells with
plasmid pIG.E1A.E1B. Transformed cell lines were established
5   from well-separated foci. We were able to establish seven
clonal cell lines, which we called PER.C1, PER.C3, PER.C4,
PER.C5, PER.C6, PER.C8 and PER.C9. One of the PER clones,
namely PER.C6, has been deposited at the ECACC under number
96022940.

10

Expression of Ad5 E1A and E1B genes in transformed A549 and
PER cells

Expression of the Ad5 E1A and the 55-kDa and 21 kDa
E1B proteins in the established A549 and PER cells was
15   studied by means of Western blotting, with the use of
monoclonal antibodies (mAb). mAb M73 recognizes the E1A
products, whereas Mabs AIC6 and C1G11 are directed against
the 55-kDa and 21 kDa E1B proteins, respectively. The
antibodies did not recognize proteins in extracts from the
20   parental A549 or the primary HER cells (data not shown). None
of the A549 clones that were generated by co-transfection of
pIG.NEO and pIG.E1A.E1B expressed detectable levels of E1A or
E1B proteins(not shown). Some of the A549 clones that were
generated by transfection with pIG.E1A.NEO expressed the Ad5
25   E1A proteins (Fig. 7), but the levels were much lower than
those detected in protein lysates from 293 cells. The steady
state E1A levels detected in protein extracts from PER cells
were much higher than those detected in extracts from A549-
derived cells. All PER cell lines expressed similar levels of
30   E1A proteins (Fig. 7). The expression of the E1B proteins,
particularly in the case of E1B 55 kDa, was more variable.
Compared to 911 and 293, the majority of the PER clones
express high levels of E1B 55 kDa and 2 kDa. The steady state
level of E1B 21 kDa was the highest in PER.C3. None of the
35   PER clones lost expression of the Ad5 E1 genes upon serial
passage of the cells (not shown). We found that the level of

E1 expression in PER cells remained stable for at least 100
population doublings. We decided to characterize the PER
clones in more detail.

5   Southern analysis of PER clones
          To study the arrangement of the Ad5-E1 encoding
sequences in the PER clones we performed Southern analyses.
Cellular DNA was extracted from all PER clones, and from 293
and 911 cells. The DNA was digested with *HindIII*, which cuts
10   once in the Ad5 E1 region. Southern hybridization on *HindIII*-
digested DNA, using a radiolabeled Ad5-E1-specific probe
revealed the presence of several integrated copies of
pIG.E1A.E1B in the genome of the PER clones. Figure 8 shows
the distribution pattern of E1 sequences in the high
15   molecular weight DNA of the different PER cell lines. The
copies are concentrated in a single band, which suggests that
they are integrated as tandem repeats. In the case of PER.C3,
C5, C6 and C9 we found additional hybridizing bands of low
molecular weight that indicate the presence of truncated
20   copies of pIG.E1A.E1B. The number of copies was determined
with the use of a Phosphor-Imager. We estimated that PER.C1,
C3, C4, C5, C6, C8 and C9 contain 2, 88, 5, 4, 5, 5, and 3
copies of the Ad5 E1 coding region, respectively, and that
911 and 293 cells contain 1 and 4 copies of the Ad5 E1
25   sequences, respectively.

Transfection efficiency
          Recombinant adenovectors are generated by co-
transfection of adapter plasmids and the large *ClaI* fragment
30   of Ad5 into 293 cells (EP application 95202213). The
recombinant virus DNA is formed by homologous recombination
between the homologous viral sequences that are present in
the plasmid and the adenovirus DNA. The efficacy of this
method, as well as that of alternative strategies, is highly
35   dependent on the transfectability of the helper cells.
Therefore, we compared the transfection efficiencies of some

60

of the PER clones with 911 cells, using the *E. coli* β-galactosidase-encoding LacZ gene as a reporter (Fig. 9).

Production of recombinant adenovirus

5   Yields of recombinant adenovirus obtained after inoculation of 293, 911, PER.C3, PER.C5 and PER.C6 with different adenovirus vectors are presented in Table II.

The results indicate that the recombinant adenovirus vector yields obtained with PER cells are at least as high as

10   those obtained with the existing cell lines. In addition, the yields of the novel adenovirus vector IG.Ad.MLPI.TK are similar or higher dm the yields obtained for the other viral vectors on all cell lines tested.

15   Generation of new adenovirus vectors (Fig. 10)

The recombinant adenovirus vectors used *(see* patent application EP 95202213) are deleted for El sequences from 459 to nt. 3328. As construct pE1A.E1B contains Ad5 sequences 459 to nt. 3510 there is a sequence overlap of 183 nt.

20   between E1B sequences in the packaging construct pIG.E1A.E1B and recombinant adenoviruses, such as for example IG.Ad.MLP.TK. The overlapping sequences were deleted from the new adenovirus vectors. In addition, noncoding sequences derived from LacZ, that are present in the original

25   constructs, were deleted as well. This was achieved *(see* Fig. 10) by PCR amplification of the SV40 poly (A) sequences from pMLP.TK using primers SV40-1 (introduces a *Bam*HI site) and SV40-2 (introduces a *Bgl*II site). In addition, Ad5 sequences present in this construct were amplified from nt. 2496 (Ad5-

30   1, introduces a *Bgl*II site) to nt. 2779 (Ad5-2). Both PCR fragments were digested with *Bgl*II and were ligated. The ligation product was PCR amplified using primers SV40-1 and Ad5-2. The PCR product obtained was cut with *Bam*HI and *Afl*II and was ligated into pMLP.TK predigested with the same

enzymes. The resulting construct, named pMLPI.TK, contains a deletion in adenovirus E1 sequences from nt. 459 to nt. 3510.

Packaging system

5

The combination of the new packaging construct pIG.E1A.E1B and the recombinant adenovirus pMLPI.TK, which do not have any sequence overlap, are presented in Fig. 11. In this figure also the original situation is presented, where

10   the sequence overlap is indicated. The absence of overlapping sequences between pIG.E1A.E1B and pMLPI.TK (Fig. 11a) excludes the possibility of homologous recombination between the packaging construct and the recombinant virus, and is therefore a significant improvement for production of

15   recombinant adenovirus as compared to the original situation.
          In Fig. 11b the situation is depicted for pIG.E1A.NEO and IG.Ad.MLPI.TK. pIG.E1A.NEO when transfected into established cells, is expected to be sufficient to support propagation of E1-deleted recombinant adenovirus. This

20   combination does not have any sequence overlap, preventing generation of RCA by homologous recombination. In addition, this convenient packaging system allows the propagation of recombinant adenoviruses that are deleted just for E1A sequences and not for E1B sequences.

25   Recombinant adenoviruses expressing E1B in the absence of E1A are attractive, as the E1B protein, in particular E1B 19 kD, is able to prevent infected human cells from lysis by Tumor Necrosis Factor (TNF) Gooding et al, (1991) J. Virol. 65:3083-3094).

30

Generation of recombinant adenovirus derived from pMLPI.TK
          Recombinant adenovirus was generated by co-transfection of 293 cells with SalI linearized pMLPI.TK DNA and ClaI linearized Ad5 wt DNA. The procedure is

35   schematically represented in Fig. 12.

Example 2

Plasmid-based system for rapid RCA-free
generation of recombinant adenoviral vectors

5

A. Construction of adenovirus clones
pBr/Ad.Bam-rITR (ECACC deposit P970821212

In order to facilitate blunt end cloning of the ITR
sequences, wild-type human adenovirus type 5 (Ad5) DNA was
10 treated with Klenow enzyme in the presence of excess dNTPs.
After inactivation of the Klenow enzyme and purification by
phenol/chloroform extraction followed by ethanol
precipitation, the DNA was digested with BamHI. This DNA
preparation was used without further purification in a
15 ligation reaction with pBr322 derived vector DNA prepared as
follows: pBr322 DNA was digested with EcoRV and BamHI,
dephosphorylated by treatment with TSAP enzyme (Life
Technologies) and purified on LNP agarose gel (SeaPlaque
GTG). After transformation into competent E. coli DH5α (Life
20 Techn.) and analysis of ampicillin resistant colonies, one
clone was selected that showed a digestion pattern as
expected for an insert extending from the BamHI site in Ad5
to the right ITR. Sequence analysis of the cloning border at
the right ITR revealed that the most 3' G residue of the ITR
25 was missing, the remainder of the ITR was found to be
correct. Said missing G residue is complemented by the other
ITR during replication.

pBr/Ad.Sal-rITR (ECACC deposit P97082119)
30   pBr/Ad.Bam-rITR was digested with BamHI and SalI. The
vector fragment including the adenovirus insert was isolated
in LMP agarose (SeaPlaque GTG) and ligated to a 4.8 kb
Sal/BamHI fragment obtained from wt Ad5 DNA and purified with
the Geneclean II kit (Bio 101, Inc.). One clone was chosen

63

and the integrity of the Ad5 sequences was determined by restriction enzyme analysis. Clone pBr/Ad.Sal-rITR contains adeno type 5 sequences from the *Sal*I site at bp 16746 up to and including the rITR (missing the most 3'G residue).

5

pBr/Ad.Cla-Bam (ECACC deposit P97082117)

wt Adeno type 5 DNA was digested with *Cla*I and *Bam*HI, and the 20.6 kb fragment was isolated from gel by electro-elution. pBr322 was digested with the same enzymes and

10 purified from agarose gel by Geneclean. Both fragments were ligated and transformed into competent DH5α. The resulting clone pBr/Ad.Cla-Bam was analyzed by restriction enzyme digestion and shown to contain an insert with adenovirus sequences from bp 919 to 21566.

15

pBr/Ad.AfII-Bam (ECACC deposit P97082114)

Clone pBr/Ad.Cla-Bam was linearized with *Eco*RI (in pBr322) and partially digested with *Afl*II. After heat inactivation of *Afl*II for 20 minutes at 65°C, the fragment

20 ends were filled in with Klenow enzyme. The DNA was then ligated to a blunt double stranded oligo linker containing a PacI site (5'-AATTGTCTTAATTAACCGCTTAA-3'). This linker was made by annealing the following two oligonucleotides: 5'-AATTGTCTTAATTAACCGC-3' and 5'-AATTGCGGTTAATTAAGAC-3',

25 followed by blunting with Klenow enzyme. After precipitation of the ligated DNA to change buffer, the ligations were digested with an excess *Pac*I enzyme to remove concatameres of the oligo. The 22016 bp partial fragment containing Ad5 sequences from bp 3534 up to 21566 and the vector sequences,

30 was isolated in LMP agarose (SeaPlaque GTG), religated and transformed into competent DH5α. One clone that was found to contain the *Pac*I site and that had retained the large adeno fragment was selected and sequenced at the 5' end to verify correct insertion of the *Pac*I linker in the (lost) *Afl*II

35 site.

pBr/Ad.Bam-rITRpac#2 (ECACC deposit P97082120) and
pBr/Ad.Bam-rITR#8 (ECACC deposit P97082121)

To allow insertion of a *PacI* site near the ITR of Ad5
in clone pBr/Ad.Bam-rITR about 190 nucleotides were removed

5   between the *ClaI* site in the pBr322 backbone and the start of
the ITR sequences. This was done as follows: pBr/Ad.Bam-rITR
was digested with *ClaI* and treated with nuclease Bal31 for
varying lengths of time (2', 5', 10' and 15'). The extent of
nucleotide removal was followed by separate reactions on

10 pBr322 DNA (also digested at the *ClaI* site), using identical
buffers and conditions. *Bal31* enzyme was inactivated by
incubation at 75°C for 10 minutes, the DNA was precipitated
and resuspended in a smaller volume TE buffer. To ensure
blunt ends, DNAs were further treated with T4 DNA polymerase

15 in the presence of excess dNTPs. After digestion of the
(control) pBr322 DNA with *SalI*, satisfactory degradation
(~150 bp) was observed in the samples treated for 10 minutes
or 15 minutes. The 10 minutes or 15 minutes treated
pBr/Ad.Bam-rITR samples were then ligated to the above

20 described blunted *PacI* linkers (*see* pBr/Ad.*AfIII*-Bam).
Ligations were purified by precipitation, digested with
excess *PacI* and separated from the linkers on an LMP agarose
gel. After religation, DNAs were transformed into competent
DH5a and colonies analyzed. Ten clones were selected that

25 showed a deletion of approximately the desired length and
these were further analyzed by T-track sequencing (T7
sequencing kit, Pharmacia Biotech). Two clones were found
with the *PacI* linker inserted just downstream of the RITR.
After digestion with *PacI*, clone #2 has 28 bp and clone #8

30 has 27 bp attached to the ITR.

pWE/Ad.AflII-rITR (ECACC deposit P97082116)

Cosmid vector pWE15 (Clontech) was used to clone
larger Ad5 inserts. First, a linker containing a unique *PacI*

35 site was inserted in the *EcoRI* sites of pWE15 creating

pWE.pac. To this end, the double stranded *PacI* oligo as
described for pBr/Ad.AflII-BamHI was used but now with its
EcoRI protruding ends. The following fragments were then
isolated by electro-elution from agarose gel:pWE.pac digested

5   with *PacI*, pBr/AflII-Bam digested with *PacI* and *BamHI* and
pBr/Ad.Bam-rITR#2 digested with *BamHI* and *PacI*. These
fragments were listed together and packaged using λ phage
packaging extracts (Stratagene) according to the
manufacturer's protocol. After infection into host bacteria,

10   colonies were grown on plates and analyzed for presence of
the complete insert. pWE/Ad.AflII-rITR contains all
adenovirus type 5 sequences, from bp 3534 (*AflII* site) up to
and including the right ITR (missing the most 3' G residue).

15   pBr/Ad.IITR-Sal(9.4) (ECACC deposit P97082115)
     Adeno 5 wt DNA was treated with Klenow enzyme in the
presence of excess dNTPs and subsequently digested with *SalI*.
Two of the resulting fragments, designated left ITR-Sal(9.4)
and Sal(16.7)-right ITR, respectively, were isolated in LMP

20   agarose (Seaplaque GTG). pBr322 DNA was digested with *EcoRV*
and *SalI* and treated with phosphatase (Life Technologies).
The vector fragment was isolated using the Geneclean method
(BIO 101, Inc.) and ligated to the Ad5 *SalI* fragments. Only
the ligation with the 9.4 kb fragment gave colonies with an

25   insert. After analysis and sequencing of the cloning border a
clone was chosen that contained the full ITR sequence and
extended to the *SalI* site at bp 9462.

             pBr/Ad.1ITR-Sal(16.7) (ECACC deposit P97082118)
30   pBr/Ad1ITR-Sal(9.4) is digested with *SalI* and
dephosphorylated (TSAP, Life Technologies). To extend this
clone up to the third *SalI* site in Ad5, pBr/Ad.Cla-Bam was
linearized with *BamHI* and partially digested with *SalI*. A 7.3
kb *SalI* fragment containing adenovirus sequences from 9462-

16746 was isolated in LMP agarose gel and ligated to the *SalI* digested pBr/Ad.IITR-Sal(9.4) vector fragment.

pWE/Ad.AflII-EcoRI

5   pWE.pac was digested with *ClaI* and the 5' protruding ends were filled in using Klenow enzyme. The DNA was then digested with *PacI* and isolated from agarose gel. pWE/AflII-rITR was digested with *EcoRI* and after treatment with Klenow enzyme digested with *PacI*. The large 24 kb fragment

10 containing the adenoviral sequences was isolated from agarose gel and ligated to the *ClaI*-digested and blunted pWE.pac vector using the Ligation Express™ kit from Clontech. After transformation of Ultracompetent XL10-Gold cells from Stratagene, clones were identified that contained the

15 expected insert. pWE/AflII-EcoRI contains Ad5 sequences from bp 3534-27336.

B.   Construction of new adapter plasmids

    The absence of sequence overlap between the

20 recombinant adenovirus and E1 sequences in the packaging cell line is essential for safe, RCA-free generation and propagation of new recombinant viruses. The adapter plasmid pMLPI.TK (Fig. 10) is an example of an adapter plasmid designed for use according to the invention in combination

25 with the improved packaging cell lines of the invention. This plasmid was used as the starting material to make a new vector in which nucleic acid molecules comprising specific promoter and gene sequences can be easily exchanged.

    First, a PCR fragment was generated from

30 pZipΔMo+PyF101(N⁻) template DNA (described in PCT/NL96/00195) with the following primers: LTR-1: 5'-CTG TAC GTA CCA GTG CAC TGG CCT AGG CAT GGA AAA ATA CAT AAC TG-3' and LTR-2: 5'-GCG GAT CCT TCG AAC CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG GCG ACT CAG TCA ATC G-3'. Pwo DNA polymerase (Boehringer

35 Mannheim) was used according to the manufacturer's protocol with the following temperature cycles: once 5 minutes at

95°C; 3 minutes at 55°C; and 1 minute at 72°C, and 30 cycles of 1 minute at 95°C, 1 minute at 60°C, 1 minute at 72°C, followed by once 10 minutes at 72°C. The PCR product was then digested with BamHI and ligated into a pMLP10 (Levrero et al,
5   (1991) Gene 101: 195-202) vector digested with PvuII and BamHI, thereby generating vector pLTR10. This vector. contains adenoviral sequences from bp 1 up to bp 454 followed by a promoter which includes part of the Mo-MuLV LTR in which the wild-type enhancer sequences are replaced by the enhancer
10   from a mutant polyoma virus (PyF101). The promoter fragment was designated L420.
        Next, the coding region of the murine HSA gene was inserted. pLTR10 was digested with BstBI followed by Klenow treatment and digestion with NcoI. The HSA gene was obtained
15   by PCR amplification on pUC18-HSA (Kay et al, (1990) J. Immunol. 145:1952-1959) using the following primers: HSA1,5'-GCG CCA CCA TGG GCA GAG CGA TGG TGG C-3' and HSA2,5'-GTT AGA TCT AAG CTT GTC GAC ATC GAT CTA CTA ACA GTA GAG ATG TAG AA-3'. The 269 bp amplified fragment was subcloned in a shuttle
20   vector using the NcoI and BglII sites. Sequencing confirmed incorporation of the correct coding sequence of the HSA gene, but with an extra TAG insertion directly following the TAG stop codon. The coding region of the HSA gene, including the TAG duplication was then excised as a NcoI(sticky)-
25   SalI(blunt) fragment and cloned into the 3.5 kb NcoI(sticky)/BstBI(blunt) fragment from pLTR10, resulting in pLTR-HSA10.
        Finally, pLTR-HSA10 was digested with EcoRI and BamHI after which the fragment containing the left ITR, packaging
30   signal, L420 promoter and HSA gene was inserted into vector pMLPI.TK digested with the same enzymes, thereby replacing the promoter and the gene sequences. This resulted in the new adapter plasmid pAd/L420-HAS (Fig. 21) that contains convenient recognition sites for various restriction enzymes
35   around the promoter and gene sequences. SnaBI and AvrII can

be combined with *HpaI*, *NheI*, *KpnI*, *HindIII* to exchange promoter sequences, while the latter sites can be combined with the *ClaI* or *BamHI* sites 3' from the HSA coding region to replace genes in this construct.

Another adapter plasmid that was designed to allow easy exchange of nucleic acid molecules was made by replacing the promoter, gene and poly A sequences in pAd/L420-HSA with the CMV promoter, a multiple cloning site, an intron and a poly-A signal. For this purpose, pAd/L420-HSA was digested with *AvrII* and *BglII*, followed by treatment with Klenow to obtain blunt ends. The 5.1 kb fragment with pBr322 vector and adenoviral sequences was isolated and ligated to a blunt 1570 bp fragment from pcDNA1/amp (Invitrogen) obtained by digestion with *HhaI* and *AvrII* followed by treatment with T4 DNA polymerase. This adapter plasmid was named pCLIP (Fig. 22).

C. Generation of recombinant adenoviruses

E1-deleted recombinant adenoviruses with wt E3 sequences

To generate E1 deleted recombinant adenoviruses with the new plasmid-based system, the following constructs were prepared: an adapter construct containing the expression cassette with the gene of interest linearized with a restriction enzyme that cuts at the 3' side of the overlapping adenoviral genome fragment, preferably not containing any pBr322 vector sequences; and a complementing adenoviral genome construct pWE/Ad.AflII-rITR digested with *PacI*.

These two DNA molecules are further purified by phenol/chloroform e and ETOH precipitation. Co-transfection of these plasmids into an adenovirus packaging cell line, preferably a cell line according to the invention, generates recombinant replication deficient adenoviruses by a one-step homologous recombination between the adapter and the

complementing construct (Fig. 23). Alternatively, instead of
pWE/Ad.AflII-rITR other fragments can be used, e.g.
pBr/Ad.Cla-Bam digested with *EcoRI* and *Bam*HI or pBr/Ad.AflII-
BamHI digested with *PacI* and *Bam*HI can be combined with
5   pBr/Ad.Sal-rITR digested with *SalI*.

          Recombinant adenovirus can be produced following
introduction of said plasmids in said cell. It is to be
understood that those skilled in the art may use other
combinations of adapter and complementing plasmids without
10   departing from the present invention.

          A general protocol as outlined below and meant as a
non-limiting example of the present invention has been
performed to produce several recombinant adenoviruses using
various adapter plasmids and the Ad.AflII-rITR fragment.
15   Adenovirus packaging cells (PER.C6) were seeded in ~25 cm^2
flasks and the next day when they were at ~80% confluency,
were transfected with a mixture of DNA and lipofectamine
agent (Life Techn.) as described by the manufacturer.
Routinely, 40 µl lipofectamine, 4 µg adapter plasmid and 4 µg
20   of the complementing adenovirus genome fragment AflII-rITR
(or 2 µg of all three plasmids for the double homologous
recombination) were used. Under these conditions transient
transfection efficiencies of ~50% (48 hrs post transfection)
were obtained as determined with control transfections using
25   a pAd/CMV-LacZ adapter. Two days later, cells were passaged
to ~80 cm^2 flasks and further cultured. Approximately five
(for the single homologous recombination) to eleven days (for
the double homologous recombination) later a cytopathic
effect (CPE) was seen, indicating that functional adenovirus
30   has formed. Cells and medium are harvested upon full CPE and
recombinant virus is released by freeze-thawing. An extra
amplification step in a 80 cm^2 flask was routinely performed
to increase the yield since at the initial stage the titers
was found to be variable despite the occurrence of full CPE.
35   After amplification, viruses was harvested and plaque

purified on PER.C6 cells. Individual plaques was tested for viruses with active transgenes.

Four different recombinant adenoviruses, containing the human interleukin-3 gene (see Figure 1, WO88/04691), the human endothelial nitric oxide gene (Janssens et al, (1992) J. Biol. Chem. 267:14519-14522), the Tc1A transposase gene (Vos et al, (1993) Genes Dev. 7:1244-1253), or the bacterial LacZ gene (Kalderon et al, (1984) Cell 39:499-509, have been produced using this protocol. In all cases, functional adenovirus was formed and all isolated plaques contained viruses with an active transgene.

E1-deleted recombinant adenoviruses with modifications in the E3 or E4 regions

Besides replacements in the E1 region it is possible to delete the E3 region or replace part of the E3 region in the adenovirus because E3 functions are not necessary for the replication, packaging and infection of a recombinant virus. This creates the opportunity to use a larger insert or to insert more than one gene without exceeding the maximum packagable size (approximately 105% of wt genome length). This can be done, for example, by deleting part of the E3 region in the pBr/Ad.Bam-rITR clone by digestion with XbaI and religation. This removes Ad5 wt sequences 28592-30470 including all known E3 coding regions. Another example is the precise replacement of the coding region of gp19K in the E3 region with a polylinker allowing insertion of new sequences. This leaves all other coding regions intact, obviates the need for a heterologous promoter since the transgene is driven by the E3 promoter and pA sequences, leaving more space for coding sequences and results in very high transgene expression, at least as good as in a control E1 replacement vector.

To this end, the 2.7 kb EcoRI fragment from wt Ad5 containing the 5' part of the E3 region was cloned into the

*Eco*RI site of pBluescript (KS⁻) (Stratagene). Next, the
*Hind*III site in the polylinker was removed by digestion with
*Eco*RV and *Hinc*II and subsequent religation. The resulting
clone pBS.Eco-Eco/ad5ΔHIII was used to delete the gp19K

5   coding region. Primers 1 (5'-GGG TAT TAG GCC **AA** AGG CGC A-3')
and 2 (5'-GAT CCC ATG GAA GCT TGG GTG GCG ACC CCA GCG-3')
were used to amplify a sequence from pBS.Eco-Eco/ad5ΔHIII
corresponding to sequences 28511 to 28734 in wt Ad5 DNA.
Primers 3 (5'-GAT CCC ATG GGG ATC CTT TAC TAA GTT ACA AAG

10   CTA-3') and 4 (5'-GTC GCT GTA GTT GGA CTG G-3') were used on
the same DNA to amplify Ad5 sequences from 29217 to 29476.
The two resulting PCR fragments were ligated together by
virtue of the newly introduced *Nco*I site and subsequently
digested with *Xba*I and *Mun*I. This fragment was then ligated

15   into a pBS.EcoEco/ad5ΔHIII vector that had been partially
digested with *Xba*I and *Mun*I, generating
pBS.EcoEco/ad5ΔHIII.Δgp19K.

     To allow insertion of foreign genes into the *Hind*III
and *Bam*HI site, an *Xba*I deletion was made in

20   pBS.Eco-Eco/ad5ΔHIII.Δgp19K to remove the *Bam*HI sites in the
Bluescript polylinker. The resulting plasmid
pBS.Eco-Eco/ad5ΔHIII.Δgp19KΔXbaI, contains unique *Hind*III
and *Bam*HI sites corresponding to sequences 28733 (*Hind*III)
and 29218 *(Bam*HI) in Ad5. After introduction of a foreign

25   gene into these sites, either the deleted *Xba*I fragment is
re-introduced, or the insert is recloned into pBS.Eco-
Eco/ad5ΔHIII.Δgp19K using *Hind*III and, for example *Mun*I.
Using this procedure, we have generated plasmids expressing
HSV-TK (McKnight (1980) *Nucl. Acid. Res.* 8:5949-5964 and

30   Vincent *et al* (1996) *Hum. Gene Ther.* 7:197-205), hIL-1α
(Esandi *et al*, (1998) *Gene Therapy* 5:778-788), rat IL-3β
(Esandi *et al*, (1998) *Gene* 211(1):151-158:), luciferase (De
Wit *et al*, (1987) *Mol. Cell Biol.* 7:725-737) or LacZ. The
unique *Srl*I and *Not*I sites in the pBS.Eco-Eco/ad5ΔHIII.Δgp19K

plasmid (with or without an inserted gene of interest) are used to transfer the region containing the gene of interest into the corresponding region of pBr/Ad.Bam-rITR, yielding construct pBr/Ad.Bam-rITRΔgp19K (with or without an inserted

5   gene of interest). This construct is used as described *supra* to produce recombinant adenoviruses. In the viral context, expression of inserted genes is driven by the adenovirus E3 promoter.

Recombinant viruses that are both E1 and E3 deleted

10   are generated by a double homologous recombination procedure as described above for E1-replacement vectors using a plasmid-based system which includes: an adapter plasmid for E1 replacement according to the invention, with or without insertion of a first gene of interest, the pWE/Ad-AflII-EcoRI

15   fragment, and the pBr/Ad.Bam-rITRΔgp19K plasmid with or without insertion of a second gene of interest.

In a non-limiting example we describe the generation and functionality of a recombinant adenovirus containing the murine HSA gene in the E1 region and the firefly luciferase

20   gene in the gp19K region. The luciferase gene was excised from pAd/MLP-Luc (described in EP 0707071) as a *HindIII-BamHI* construct and cloned into the *HindIII-BamHI* sites of pBS.Eco-Eco/ad5ΔHIII.Δgp19KΔXbaI. Then the *MscI-MunI* fragment containing the luciferase gene was cloned into the

25   corresponding sites of pBS.Eco-Eco/ad5Δgp19K generating pBS.Eco-Eco/ad5Δgp19K.luc. This restores the Eco-Eco fragment, but now with the luciferase gene in the place of gp19K.

To simplify further manipulation, the internal *EcoRI*

30   sites in the luciferase insert were mutated without making changes to the amino acid sequence of the luciferase gene. One *EcoRI* site flanked the *HindIII* site in the 5'non-coding region of the luciferase insert and the other one was located 588 bp 3' from the starting ATG. A 695 bp PCR product was

35   generated with the following primers: 5'-CGA TAA GCT TAA TTC

CTT TGT GTT T-3' and 5'-CTT AGG TAA CCC AGT AGA TCC AGA GGA
GTT CAT-3' and digested with *Hind*III and *Bst*EII. This
fragment was then ligated to *Hind*III-*Bst*EII digested
pBS.Eco-Eco/ad5Δgp19K.luc, replacing the corresponding insert
5 in this vector. The resulting construct is named
pBS.EcoEco/ad5Δgp19K.luc². The luciferase gene and part of
the E3 region was then excised from this clone with *Srf*I and
*Not*I and introduced in the corresponding sites in pBr/Ad.Bam-
rITR generating clone pBr/Ad.Bam-rITRΔgp19K/luc².

10   The adapter plasmid pAd5/SI1800HSA used for the
replacement of E1 in the double insert virus contains the
murine HSA gene driven by a retrovirus LTR-based promoter.
This adapter plasmid was generated from the pAd5/L420-HSA
construct described *infra* by replacement of the promoter
15 sequence. First a PCR product was generated on a retroviral
vector based on the MFG-S vector described in WO95/34669
using the same primers as for the amplification of the L420
promoter fragment (described *infra*). This PCR amplifies the
sequences corresponding to bp 453-877 in the MFG-S vector.
20 The L420 promoter in pAd5/L420-HSA **(Fig. 21)** was then
exchanged for the PCR fragment using the unique *Avr*II and
*Hind*III sites. The resulting construct, pAd5/S430-HSA, was
then digested with *Nhe*I and *Sca*I and the 4504 bp fragment
containing the HSA gene, pA sequences, Ad5 sequences and
25 vector sequences to the *Sca*I site in the ampicillin gene was
isolated.
         The construct pAd5/S430-HSA also was digested with
*Xba*I and *Sca*I and the 1252 bp fragment (containing the
remainder of the ampicillin gene, the left ITR and packaging
30 signal from adenovirus and the 5' part of the S430 promoter)
was isolated. A third fragment of 1576 bp was isolated from
the MFG-S-based retroviral vector following an *Xba*I digestion
and contains MFG-S sequences corresponding to bp 695-2271.
         The adapter plasmid pAd5/S1800-HSA was constructed by
35 ligating the three isolated fragments. The double insert

virus Ad5/S1800-HSA.E3luc was generated (as described above) by transfection of the following DNA fragments into PER.C6 cells: pAd5/S1800-HSA digested with *EcoRI and SalI* (2 µg) + pWE/Ad.AflII-EcoRI (2 µg) digested with PacI and EcoRI +

5   pBr/Ad.Bam-rITRΔgp19klac^z digested with SalI.. At occurrence of CPE, the virus was harvested and amplified by serial passages on PER.C6 cells. The activity of this HSA-Luc virus was compared to single insert ΔE1 viruses containing either the S1800-HSA or the CMV-Luc transcription units in the E1

10   region. A549 cells were seeded at 2x10^5 cells/well and infected 5 hrs later with different amounts of the virus. Two days later transgene expression was measured. Luciferase activity was measured using a luciferase assay system (Promega) and expression of the murine HSA gene was measured

15   with an α-HSA antibody (M1/69, Pharmingen). The results are fisted in Table III.
            This experiment shows that using the plasmid-based recombination system, double insert viruses can be made and that both inserts are functional. Furthermore, the luciferase

20   activity of the double insert viruses is comparable to the CMV-driven luciferase activity of the control virus. Therefore, we conclude that the E3 promoter is highly active in A549 cells, even in the absence of E1A proteins.
            In addition to manipulations in the E3 region,

25   changes of (parts of) the E4 region can be accomplished easily in pBr/Ad.Bam-rITR. Generation and propagation of such a virus, however, in some cases demands complementation *in trans*.

Example 3

Demonstration of the competence of a synthetic DNA sequence, that is capable of forming a hairpin structure, to serve as a primer for reverse strand synthesis for the generation of double-stranded DNA molecules in cells that contain and express adenovirus genes

Name convention of the plasmids used:

p	plasmid
I	ITR (Adenovirus Inverted Terminal Repeat)
C	Cytomegalovirus (CMV) Enhancer/Promoter Combination
L	Firefly Luciferase Coding Sequence
	hac, haw Potential hairpin that can be formed after digestion with restriction endonuclease Asp718 in both the correct and in the reverse orientation, respectively (Fig. 15).

The naming convention is exemplified as follows. pICLhaw is a plasmid that contains the adenovirus ITR followed by the CMV-driven luciferase gene and the Asp718 hairpin in the reverse (non-functional) orientation. Plasmids pICLhac, pICLhaw, pICLI and pICL were generated using standard techniques. The schematic representation of these plasmids is shown in Figs. 16-19.

Plasmid pICL is derived from the following plasmids:

nt. 1	457	pMLP10 (Levrero et al (1991)) Gene 101:195-202)
nt. 458	1218	pCMVβ (Clontech, EMBL Bank No. U02451)
nt. 1219	3016	pMLP.luc (IntroGene, unpublished)
nt. 3017	5620	pBLCAT5 (Stein et al, (1989) Mol. Cell Biol. 9:4531-4).

The plasmid has been constructed as follows:

The tet gene of plasmid pMLP10 has been inactivated
by deletion of the *BamHI-SalI* fragment, to generate
pBLP10ΔSB. Using primer set PCR/MLP1 and PCR/MLP3 a 210 bp
fragment containing the Ad5-ITR, flanked by a synthetic *SalI*

5   restriction site was amplified using pMLP10 DNA as the
template. The PCR product was digested with the enzymes *EcoRI*
and *SgrAI* to generate a 196 bp fragment. Plasmid pMLP10ΔSB
was digested with *EcoRI* and *SgrAI* to remove the ITR. This
fragment was replaced by the *EcoRI-SgrAI*-treated PCR fragment

10   to generate pMLP/SAL.

Plasmid pCMV-Luc was digested with *PvuII* to
completion and recirculated to remove the SV40-derived poly-
adenylation signal and Ad5 sequences with exception of the
Ad5 left-terminus. In the resulting plasmid, pCMV-LucΔAd, the

15   Ad5 ITR was replaced by the Sal-site-flanked ITR from plasmid
pMLP/SAL by exchanging the *XmnI-SacII* fragments. The
resulting plasmid, pCMV-LucΔAd/SAL, the Ad5 left terminus and
the CMV-driven luciferase gene were isolated as a *SalI-SmaI*
fragment and inserted in the *SalI* and *HpaI* digested plasmid

20   pBLCATS, to form plasmid pICL. Plasmid pICL is represented in
Fig. 19; its sequence is presented in Fig. 20.

Plasmid pICL contains the following features:

nt.1-457   Ad5 left terminus (Sequence 1-457 of
            human adenovirus type 5)

25   nt.458-969   Human cytomegalovirus enhancer and
                          immediate early promoter (Boshart *et al*,
                          (1985) *Cell* 41:521-530) (from plasmid
                          pCMVβ, Clontech, Palo Alto, USA)

            nt.970-1204   SV40 19S exon and truncated 16/19S
30   intron (from plasmid pCMVβ)

            nt.1218-2987 Firefly luciferase gene (from pMLP.luc)

            nt.3018-3131 SV40 tandem poly-adenylation signals
                          from late transcript, derived from
                          plasmid pBLCAT5)

nt.3132-5620 pUC12 backbone (derived from plasmid
pBLCAT5)
nt.4337-5191 β-lactamase gene (Amp-resistance gene,
reverse orientation)

5   Plasmids pICLhac and pICLhaw

Plasmids pICLhac and pICLhaw were derived from
plasmid pICL by digestion of pICL with the restriction enzyme
Asp718. The linearized plasmid was treated with Calf-
Intestine Alkaline Phosphatase to remove the 5¹ phosphate
10   groups. The partially complementary synthetic single-stranded
oligonucleotides Hp/asp1 and Hp/asp2 were annealed and
phosphorylated on their 5' ends using T4-polynucleotide
kinase.

The phosphorylated double-stranded oligomers were
15   mixed with the dephosphorylated pICL fragment and ligated.
Clones containing a single copy of the synthetic
oligonucleotide inserted into the plasmid were isolated and
characterized using restriction enzyme digests. Insertion of
the oligonucleotide into the Asp718 site will at one junction
20   recreate an Asp718 recognition site, whereas at the other
junction the recognition site will be disrupted. The
orientation and the integrity of the inserted oligonucleotide
was verified in selected clones by sequence analyses. A clone
containing the oligonucleotide in the correct orientation
25   (the Asp718 site close to the 3205 EcoRI site) was denoted
pICLhac. A clone with the oligonucleotide in the reverse
orientation (the Asp718 site close to the SV40 derived poly-
signal) was designated pICLhaw. Plasmids pICLhac and pICLhaw
are represented in Figs. 16 and 17.
30   Plasmid pICLI was created from plasmid pICL by
insertion of the SalI-SgrAI fragment from pICL, containing
the Ad5-ITR into the Asp718 site of pICL. The 194 bp SalI-
SgrAI fragment was isolated from pICL, and the cohesive ends
were converted to blunt ends using E. coli DNA polymerase I
35   (Klenow fragment) and dNTP's. The Asp718 cohesive ends were

converted to blunt ends by treatment with mungbean nuclease.
By ligation clones were generated that contain the ITR in the
Asp718 site of plasmid pICL. A clone that contained the ITR
fragment in the correct orientation was designated pICLI

5   (Fig. 18).
        Generation of adenovirus Ad-CMV-hcTK. Recombinant
adenovirus was constructed according to the method described
in Patent application 95202213. Two components are required
to generate a recombinant adenovirus. First, an adapter-

10 plasmid containing the left terminus of the adenovirus genome
containing the ITR and the packaging signal, an expression
cassette with the gene of interest, and a portion of the
adenovirus genome which can be used for homologous
recombination. In addition, adenovirus DNA is needed for

15 recombination with the aforementioned adapter plasmid. In the
case of Ad-CMV-hcTK, the plasmid PCMV.TK was used as a basis.
This plasmid contains nt.1-455 of the adenovirus type 5
genome, nt. 456-1204 derived from PCMVβ (Clontech, the *PstI-
StuI* fragment that contains the CMV enhancer promoter and the

20 16S/ 19S intron from simian Virus 40), the Herpes Simplex
Virus thymidine kinase gene (described in EP patent
application 95202213.5), the SV40-derived polyadenylation
signal (nt. 2533-2668 of the SV40 sequence), followed by the
BgIII-ScaI fragment of Ad5 (nt. 3328-6092 of the Ad5

25 sequence). These fragments are present in a pMLP10-derived
(Levrero et *al*, (1991) *Gene* 101: 195-202) backbone. To
generate plasmid pAD-CNWhc-TK, plasmid PCMV.TK was digested
with *ClaI* (the unique *ClaI*-site is located just upstream of
the TK open reading frame) and dephosphorylated with Calf-

30 Intestine Alkaline Phosphate. To generate a hairpin-
structure, the synthetic oligonucleotides HP/*cla2* and HP/*cla2*
were annealed and phosphorylated on their 5'-OH groups with
T4-polynucleotide kinase and ATP. The double-stranded
oligonucleotide was ligated with the linearized vector

35 fragment and used to transform E. *coli* strain "Sure".

Insertion of the oligonucleotide into the *ClaI* site will
disrupt the *ClaI* recognition sites. The oligonucleotide
contains a new *ClaI* site near one of its termini. In selected
clones, the orientation and the integrity of the inserted

5   oligonucleotide was verified by sequence analyses. A clone
containing the oligonucleotide in the-correct orientation
(the *ClaI* site at the ITR side) was denoted pAd-CMV-hcTK.
This plasmid was co-transfected with *ClaI*-digested wild-type
adenovirus-type 5 DNA into 911 cells. A recombinant

10   adenovirus in which the CMV-hcTK expression cassette replaces
the E1 sequences was isolated and propagated using standard
procedures.

To study whether the hairpin can be used as a primer
for reverse strand synthesis on the displaced strand after

15   replication has started at the ITR, the plasmid piCLhac was
introduced into 911 cells, i.e. human embryonic retinoblasts
transformed with the adenovirus-E1 region. The plasmid
pICLhaw served as a control: it contains the oligonucleotide
pair HP/asp 1 and 2 in the reverse orientation but is

20   otherwise completely identical to plasmid piCLhac. Also
included in these studies were plasmids pICLI and pICL. In
the plasmid pICLI the hairpin is replaced by an adenovirus
ITR. Plasmid pICL contains neither a hairpin nor an ITR
sequence. These plasmids served as controls to determine the

25   efficiency of replication by virtue of the terminal hairpin
structure. To provide the viral products other than the E1
proteins (these are produced by the 911 cells) required for
DNA replication the cultures were infected with the virus
IG.Ad.MLPI.TK after transfection. Several parameters were

30   being studied to demonstrate proper replication of the
transfected DNA molecules. First, DNA extracted from the cell
cultures transfected with the aforementioned plasmids and
infected with IG.Ad.MLPI.TK virus was analyzed by Southern
blotting for the presence of the expected replication

35   intermediates, as well as for the presence of the duplicated
genomes. Furthermore, from the transfected and IG.Ad.hMPI.TK

infected cell populations, virus was isolated that can
transfer a luciferase marker gene into luciferase negative
cells and express it.

5   Plasmid DNA of plasmids pICLhac, pCLhaw, pICLI and
PICL were digested with restriction endonuclease *SalI* and
treated with mungbean nuclease to remove the 4 nucleotide
single-stranded extension of the resulting DNA fragment. In
this manner a natural adenovirus 5' ITR terminus on the DNA
fragment was created. Subsequently, both the pICLhac and
10   pICLhaw plasmids were digested with restriction endonuclease
*Asp*718 to generate the terminus capable. of forming a hairpin
structure. The digested plasmids were introduced into 911-
cells, using the standard calcium phosphate co-precipitation
technique, four dishes for each plasmid. During the
15   transfection, for each plasmid two of the cultures were
infected with the IG.Ad.MLPI.TK virus using 5 infectious
IG.Ad.MLPI.TK particles per cell. At twenty-hours post
transfection and forty hours post-transfection one Ad.tk-
virus-infected and one uninfected culture were used to
20   isolate low molecular-weight DNA using the procedure devised
by Hirt (as described in Einerhand et al, (1995) *Gene Therapy*
2:336-343). Aliquots of isolated DNA were used for Southern
analysis. After digestion of the samples with restriction
endonuclease EcoRI using the luciferase gene as a probe a
25   hybridizing fragment of approx. 2.6kb were detected in only
the samples from the adenovirus-infected cells transfected
with plasmid pICLhac. The size of this fragment was
consistent with the anticipated duplication of the luciferase
marker gene. This supports the conclusion that the inserted
30   hairpin is capable of serving as a primer for reverse stand
synthesis. The hybridizing fragment was absent if the
IG.Ad.MLPI.TK virus was omitted, or if the hairpin
oligonucleotide was inserted in the reverse orientation.

The restriction endonuclease Dpnl recognizes the
35   tetranucleotide sequence 5'-GATC-3', but cleaves only
methylated DNA, (that is, only plasmid DNA propagated in, and

derived, from *E. coli*, not DNA that has been replicated in
mammalian cells). The restriction endonuclease *Mbo*I
recognizes the same sequences, but cleaves only unmethylated
DNA (namely, DNA propagated in mammalian cells). DNA samples

5   isolated from the transfected cells are incubated with *Mbo*I
and *Dpn*I and analyzed with Southern blots. These results
demonstrated that only in the cells transfected with the
pICLhac and the pICLI plasmids large *Dpn*I-resistant fragments
were present, that were absent in the *Mbo*I treated samples.

10 These data demonstrate that only after transfection of
plasmids pICLI and pICLhac replication and duplication of the
fragments occur.

       These data demonstrate that in adenovirus-infected
cells linear DNA fragments that have on one terminus an

15 adenovirus-derived inverted terminal repeat (ITR) and at the
other terminus a nucleotide sequence that can anneal to
sequences on the same strand, when present in single-stranded
form thereby generate a hairpin structure, and will be
converted to structures that have inverted terminal repeat

20 sequences on both ends. The resulting DNA molecules will
replicate by the same mechanism as the wild-type adenovirus
genomes.

                          <u>Example 4</u>

       <u>Demonstration that the DNA molecules that contain a</u>

25 <u>luciferase marker gene, a single copy of the ITR, the</u>
    <u>encapsidation signal and a synthetic DNA sequence, that is</u>
    <u>able of forming a hairpin structure, are sufficient to</u>
    <u>generate DNA molecules that can be encapsidated into virions</u>

30   To demonstrate that the DNA molecules, generated in
Example 3, containing two copies of the CMV-Luc marker gene
can be encapsulated into virions, virus was harvested from
the remaining two cultures via three cycles of freeze-thaw
crushing and was used to infect murine fibroblasts. Forty-

82

eight hours after infection the infected cells are assayed
for luciferase activity. To exclude the possibility that the
luciferase activity has been induced by transfer of free DNA,
rather than *via* virus particles, virus stocks were treated

5   with DNaseI to remove DNA contaminants. Furthermore, as an
additional control, aliquots of the virus stocks were
incubated for 60 minutes at 56°C. The heat treatment does not
affect the contaminating DNA, but does inactivate the
viruses. Significant luciferase activity was only found in

10 the cells after infection with the virus stocks derived from
IG.Ad.MLPI.TK-infected cells transfected with the pICLhc and
PICLI plasmids. Neither in the non-infected cells, nor in the
infected cells transfected with the pICLhw and pICL was
significant luciferase activity demonstrated. Heat

15 inactivation, but not DNaseI treatment, completely eliminated
luciferase expression, demonstrating that adenovirus
particles, and not free (contaminating) DNA fragments were
responsible for transfer of the luciferase reporter gene.
        These results demonstrate that these small viral

20 genomes can be encapsulated into adenovirus particles and
suggest that the ITR and the encapsulation signal are
sufficient for encapsulation of linear DNA fragments into
adenovirus particles. These adenovirus particles can be used
for efficient gene transfer. When introduced into cells that

25 contain and express at least some of the adenovirus genes
(namely E1, E2, E4, and L, and VA), recombinant DNA molecules
that include at least one ITR, at least part of the
encapsulation signal as well as a synthetic DNA sequence,
that is capable of forming a hairpin structure, have the

30 intrinsic capacity to autonomously generate recombinant
genomes which can be encapsulated into virions. Such genomes
and vector system can be used for gene transfer.

Example 5

<u>Demonstration that DNA molecules which contain nucleotides</u>
<u>3510-35953 (namely 9.7-100 may units) of the adenovirus type</u>
<u>5 genome (thus lack the E1 protein-coding regions, the right-</u>
5   <u>hand ITR and the encapsidation sequences) and a terminal DNA</u>
<u>sequence that is complementary to a portion of the same</u>
<u>strand of the DNA molecule when present in single-stranded</u>
<u>form other than the ITR, and as a result is capable of</u>
<u>forming a hairpin structure, can replicate in 911 cells</u>
10

           In order to develop a replicating DNA molecule that
    can provide the adenovirus products required to allow the
    above-mentioned ICLhac vector genome and alike minimal
    adenovectors to be encapsulated into adenovirus particles by
15   helper cells, the Ad-CW-hcfK adenoviral vector was developed.
    Between the CMV enhancer/promoter region and the thymidine
    kinase gene, the annealed oligonucleotide pair (Table 1)
    HP/cla 1 and 2 was inserted. The vector Ad-CMV-hcTK was
    propagated and produced in 911 cell using standard
20   procedures. This vector was grown and propagated exclusively
    as a source of DNA used for transfection. DNA of the
    adenovirus AdCMV-hcTK was isolated from virus particles that
    had been purified using CsCl density-gradient centrifugation
    by standard techniques. The virus DNA was digested with
25   restriction endonuclease ClaI. The digested DNA was size-
    fractionated on an 0.7% agarose gel and the large fragment
    was isolated and used for further experiments. Cultures of
    911 cells were transfected with the large ClaI-fragment of
    the Ad-CMV-hcTK DNA using standard calcium phosphate co-
30   precipitation techniques. Much like in the previous
    experiments with plasmid pICLhac, the Ad-CMV-hc replicates
    starting at the right-hand ITR. Once the I -strand is
    displaced, a hairpin can be formed at the left-hand terminus
    of the fragment. This facilitates DNA polymerase elongation
35   of the chain towards the right-hand side. The process

proceeds until the displaced strand is completely converted
to its double-stranded form. Finally, the right-hand ITR is
recreated, and in this location, normal adenovirus
replication-initiation and elongation occur. The polymerase
5   reads through the hairpin, thereby duplicating the molecule.
The input DNA molecule of 33250 bp, that had on one side an
adenovirus ITR sequence and at the other side a DNA sequence
that had the capacity to form a hairpin structure is
duplicated so that both ends contain an ITR sequence. The
10   resulting DNA molecule consists of a palindromic structure of
approximately 66500 bp.

          This structure is detected in low-molecular weight
DNA extracted from transfected cells using Southern analysis.
The palindromic nature of the DNA fragment can be
15   demonstrated by digestion of the low-molecular weight DNA
with suitable restriction endonucleases and Southern blotting
with the HSV-TK gene as the probe. This molecule can
replicate itself in the transfected cells by virtue of the
adenovirus gene products that are present in the cells. In
20   part, the adenovirus genes are expressed from templates that
are integrated in the genome of the target cells (namely, the
E1 gene products), the other genes reside in the replicating
DNA fragment itself. This linear DNA fragment cannot be
encapsulated into virions. Not only does it lack all the DNA
25   sequences required for encapsulation, but its size also is
much too large to be encapsulated.

Example 6

Demonstration that DNA molecules which contain nucleotides
3503-35953 (viz. 9.7-100 map units) of the adenovirus type 5
genome (thus lack the E1 protein-coding regions, the right-
hand ITR and the encapsidation sequences) and a terminal DNA
sequence that is complementary to a portion the same strand
of the DNA molecule other than the ITR, and as a result is
capable of forming a hairpin structure, can replicate in 911
cells and can provide the helper functions required to
encapsidate the pICLI and pICLhac derived DNA fragments

        The purpose of the next series of experiments is to
demonstrate that the DNA molecule described in Example 5 can
be used to encapsulate the minimal adenovectors described in
Examples 3 and 4.
        The large fragment isolated after endonuclease ClaI-
digestion of Ad-CW-hcTK DNA was introduced into 911 cells (as
described in Example 5) together with endonuclease SalI,
mungbean nuclease, endonuclease Asp718-treated plasmid
pICLhac, or as a control similarly treated plasmid pICLhaw.
After 48 hours virus was isolated by freeze-thaw crushing of
the transfected cell population. The virus preparation was
treated with DNaseI to remove contaminating free DNA. The
virus was used subsequently to infect Rat2 fibroblasts.
Forty-eight hours post infection the cells were assayed for
luciferase activity. Only in the cells infected with virus
isolated from the cells transfected with the pICLhac plasmid,
and not with the pICLhaw plasmid, was significant luciferase
activity demonstrated. Heat inactivation of the virus prior
to infection completely abolished the luciferase activity,
indicating that the luciferase gene was transfected by a
viral particle. Infection of 911 cell with the virus stock
did not result in any cytopathological effects, demonstrating
that pICLhac was produced without any infectious helper virus
being propagated on 911 cells. These results demonstrate that

the proposed method can be used to produce stocks of minimal-
adenoviral vectors, that are completely devoid of infectious
helper viruses that are able to replicate autonomously on
adenovirus-transformed human cells or on non-adenovirus

5   transformed human cells.

Example 7

Construction of plasmids for the generation and production of minimal adenoviral vectors

10   A minimal adenovirus vector contains as operably
linked components the adenovirus-derived *cis* elements
necessary for replication and packaging, with or without
foreign nucleic acid molecules to be transferred. Recently,
the lower limit for efficient packaging of adenoviral vectors

15   has been determined at 75% of the genome length (Parks and
Graham, 1997 [need cite]. To allow flexible incorporation of
various lengths of stuffer fragments, a multiple cloning site
(MCS) was introduced into a minimal adenoviral vector. To
obtain a minimal adenoviral vector according to the

20   invention, the following constructs were made: pAd/L420-HSA
(Fig. 21) was digested with *Bgl*II and *Sal*I and the vector-
containing fragment was isolated. This fragment contains the
left ITR and packaging signal from Ad5 and the murine HSA
gene driven by a modified retroviral LTR. The right ITR of

25   adenovirus was amplified by PCR on pBr/Ad.BamHI-rITR template
DNA using the following primers: PolyL-ITR: 5'-AAC-TGC-AGA-
TCT-ATCGAT-ACT-AGT-CAA-TTG-CTC-GAG-TCT-AGA-CTA-CGT-CAC-CCG-
CCC-CGT-TCC-3' and ITR-BSN: 5'-CGG-GAT-CCG-TCG-ACG-CGG-CCG-
CAT-CAT-CAA-TAA-TAT-ACC3'. The amplified fragment was

30   digested with *Pst*I and *Bam*HI and cloned into pUC119 digested
with the same enzymes. After sequence confirmation of correct
amplification of the ITR and the MCS, a *Bgl*II-*Sal*I fragment
was isolated and cloned into the *Bgl*II/*Sal*I-digested

pAd/L420-HSA fragment described above. The resulting clone
was named pAd/L420-HSA.ITR.

   To be able to manipulate constructs of lengths
exceeding 30 kb, the minimal adenoviral vector pAd/L420-
5   HSA.ITR was subcloned in a cosmid vector background. To this
end, the cosmid vector pWE15 was modified to remove
restriction sites in the backbone pWE15 was digested with
PstI and fragments of 4 kb and 2.36 kb were isolated from
agarose gel and ligated together. The resulting clone,
10   stripped of the SV40 ori/early promoter and neomycine
resistance coding sequence, was named pWE20. Then, pWE20 was
digested with ClaI and HindIII and the sticky ends were
filled in with Klenow enzyme. A 6354 bp blunt fragment was
ligated to a phosphorylated NsiI linker with the following
15   sequence: 5'-CGATGCATCG-3'. The ligated DNA was
phenol/chloroform extracted, precipitated with EtOH to change
buffers, and digested with excess NsiI. Digested DNA was
separated from the linkers by electrophoresis, isolated and
religated. The resulting clone was named pWE25. Correct
20   insertion of the NsiI linker was confirmed by restriction
enzyme digestion and sequencing. To construct the minimal
adenoviral vector, pAd/L420-HSA.ITR was digested with ScaI
and NotI and the 2 kb fragment containing part of the
ampicillin gene and the adeno ITRs was cloned into pWE25
25   digested with ScaI and NotI. The resulting clone was named
pMV/L420H (Fig. 24). This clone allows easy manipulation to
exchange the promoter and/or gene, and also- allows insertion
of DNA fragments of lengths not easily cloned into normal
plasmid backbones.
30   Plasmid pMV/CMV-LacZ was made by exchanging the L420-
HSA fragment (SnaBI-BamHI) for a fragment from pcDNA3-nlsLacZ
(NruI-BamHI) containing the CMV promoter and LacZ coding
sequences. pcDNA3-nlsLacZ was constructed by insertion of a
KpnI-BamHI fragment obtained after PCR amplification of the
35   nlsLacZ coding sequences into pcDNA3 (Invitrogen) digested

with *KpnI* and *BamHI*. The PCR reaction was performed on a
pMLP.nlsLacZ template DNA using the primers 1: 5'-GGG-GTG-
GCC-AGG-GTA-CCT-CTA-GGC-TTT-TGC-AA-3' and 2: 5'-GGG-GGG-ATC-
CAT-AAA-CAA-GTT-CAG-AAT-CC-3'. Correct amplification and

5   cloning were confirmed by assaying β-galactosidase expression
in a transient transfection experiment on 911 cells.
        The vector pAd/MLPnlsLacZ was made as follows: pMLP10
(Levrero *et al*, (1991) *Gene* 101:195-202) was digested with
HindIII and *BamHI* and ligated, in a three-part ligation, to a

10   3.3 kb *AvrII-BamHI* fragment from L7RHβ-gal (Kalderon *et al*,
(1984) *Cell* 499-509), and a synthetic linker with *HindIII* and
XbaI overhang. The linker was made by annealing two
oligonucleotides of sequence 5'-AGC TTG AAT TCC CGG GTA CCT-
3' and 5'-CTA GAG GTA CCC GGG AAT TCA-3'. The resulting clone

15   was named pMLP.nlsLacZ/-Ad. Next, pMLP.nlsLacZ/-Ad was
digested with *BamHI* and *NruI* and the vector containing
fragment was ligated to a 2766 bp *BglII-ScaI* fragment from
pAd5SalB (Bernards *et al*, (1982) *Virology* 120:422-432). This
resulted in the adapter plasmid pMLP.nlsLacZ (described in

20   EP 0 707 071).
        Propagation of a minimal adenoviral vector can only
be achieved by expression of adenovirus gene products.
Expression of adenovirus gene products, at levels high enough
to sustain production of large quantities of virus, requires

25   replication of the coding nucleic acid molecule. Usually,
therefore, replicating helper viruses are used to complement
the minimal adenoviral vectors. The present invention,
however, provides packaging systems for minimal adenoviral
vectors without the use of helper viruses. One of the methods

30   of the invention makes use of a replicating DNA molecule that
contains the 5'-ITR and all adenoviral sequences between bp
3510 and 35938, i.e., the complete adenoviral genome except
for the E1 region and the packaging signal. Construct
pWE/Ad.Δ5' (Fig. 25) is an example of a replicating molecule

35   according to the invention that contains two adenoviral-ITRs.

pWE/Ad.Δ5'. It has been made in a cosmid vector background
from three fragments. First, the 5' ITR from Ad5 was
amplified using the following primers:
ITR-EPH: 5'-CGG-AAT-TCT-TAA-TTA-AGT-TAA-CAT-CAT-CAA-TAA-TAT-
5   ACC-3' and
ITR-pIX: 5'-ACG-GCG-CGC-CTT-AAG-CCA-CGC-CCA-CAC-ATT-TCA-GTA-
CGT-ACT-AGT-CTA-CGT-CAC-CCG-CCC-CGT-TCC-3'. The resulting PCR
fragment was digested with EcoRI and AscI and cloned into
vector pNEB193 (New England Biolabs) digested with the same
10 enzymes. The resulting construct was named pNEB/ITR-pIX.
Sequencing confirmed correct amplification of the Ad5
sequences in the left ITR (Ad5 sequences 1 to 103) linked to
the pIX promoter (Ad5 sequences 3511 to 3538) except for a
single mismatch with the expected sequence according to
15 GenBank (Accession No.: M73260/M29978), i.e., an extra
C-residue was found just upstream of the AflII site. This
ITR-pIX fragment was isolated with EcoRI and AflII and
ligated to a EcoRI-AflII vector fragment containing Ad5
sequences 3539-21567. The latter fragment was obtained by
20 digestion of pBr/Ad.Cla-Bam (supra) with EcoRI and partially
with AflII. The resulting clone was named pAd/LITR(Δ5')-
BamHI. The final construct pWE/Ad.Δ5' was made by ligating
cosmid vector pWE15.Pac (supra) digested with PacI to
pAd/LITR(Δ5')-BamHI digested with PacI-BamHI and pBr/Ad.Bam-
25 rITR.pac#2 (supra) digested with PacI/BamHI (Fig. 25).

An alternative method to produce packaging systems
for minimal adenoviral vectors without the use of helper
viruses according to the invention is to use a replicating
DNA molecule that contains the complete adenoviral genome
30 except for the E1 region and the packaging signal and in
which one of the ITRs is replaced by a fragment containing a
DNA sequence complementary to a portion of the same strand
other than the ITR and that therefore is able to form a
hairpin structure (Fig. 10). In a non-limiting example, said
35 DNA sequence complementary to a portion of the same strand

other than the ITR is derived from the adeno-associated virus
(AAV) terminal repeat. Such a replicating DNA molecule is
made following the same cloning strategy as described for
pWE/Ad.Δ5', but now starting with the AAV terminal repeat
5   linked to part of the adenoviral pIX promoter. To this end,
the adenoviral ITR sequences between the *Hpa*I and *Spe*I sites
in construct pNEB/ITR-pIX were exchanged for the AAV ITR by
introducing the *Pvu*II/*Xba*I fragment from psub201(+)
containing the AAV ITR (Samulski *et al*, (1989) *J. Virol*.
10   63:3822-3828). This results in construct pWE/AAV.Δ5' that
replicates in an E1 complementing cell line.

        Another alternative packaging system for minimal
adenoviral vectors is described *infra* and makes use of the
replication system of SV40. A functional helper molecule
15   according to this method contains at least the adenoviral
sequences necessary to sustain packaging of a minimal
construct but not the E1 sequences and packaging signal, and
preferably also lacking ITRs. This adenovirus-derived entity
has to be present on a vector that contains, besides the
20   sequences needed for propagation in bacteria, an origin of
replication from SV40 virus. Transfection of such a molecule
together with the minimal adenoviral vector, described *supra*,
into a packaging cell line (e.g. PER.C6) expressing, besides
the E1 proteins, SV40 derived Large T antigen proteins,
25   results in Large T-dependent replication of the adenovirus-
derived helper construct. This replication leads to high
levels of adenoviral proteins necessary for replication of
the minimal adenoviral vector and packaging into virus
particles. In this way, there is no sequence overlap that
30   leads to homologous recombination between the minimal
adenoviral vector construct and the helper molecule. In
addition, there is no sequence overlap that leads to
homologous recombination between the helper molecule and
minimal adenoviral vector on the one side and the E1 sequence
35   in the packaging cell on the other side.

Replication of a 40 kb adenoviral construct was
investigated in cells expressing SV40 Large T proteins.
Hereto, 2 x 10^{6}Cos-1 cells were transfected in a T25 flask
with the following constructs complexed with lipofectamine

5　reagent (Life techn.): the 8 kb cosmid vector pWE.pac, the
40.5 kb construct pWE/Ad.AflII-rITR and three clones (#1, #5
and #9) of the 40.6 kb construct pWE/Ad.Δ5' (described
infra). Control transfections were carried out with the
constructs pWE.pac and pWE/Ad.AflII-rITR digested with PacI

10　enzyme and a CMV-LacZ expression vector without the SV40 ori
sequence. Transfection efficiency was 50% as determined by a
separate transfection using the CMV-LacZ vector and X-gal
staining after 48 hrs. All cells were harvested 48 hrs.
following transfection and DNA was extracted according to the

15　Hirt procedure (as described in Einerhand et al, (1995) Gene
Therapy 2:336-343). Final pellets were resuspended in 50 μl
TE+RNase (20 μg/ml) and 10 μl samples were digested with MboI
(35 units overnight at 37°C). Undigested samples (5 μl) and
MboI digested samples were run on a 0.8% agarose gel,

20　transferred to a nylon filter (Amersham) and hybridized to
radioactive probes according to standard procedures. One
probe was derived from an 887 bp DpnI fragment from the
cosmid vector pWE.pac and one was derived from a 1864 bp
BsrGI-BamHI fragment from adenoviral sequences. These probes

25　hybridize to a 887 bp band and a 1416 bp respectively in MboI
digested material. Input DNA from bacterial origin is
methylated and therefore not digested with MboI. In this way
it is possible to specifically detect DNA that is replicated
in eukaryotic cells. Figure 26A shows a schematic

30　presentation of the construct pWE/Ad.Δ5' and the locations of
the SV40 origin of replication, the pWE-derived probe and the
adenovirus derived probe. The lower part presents the
autoradiograms of the Southern blots hybridized to the
adenovirus probe (B) and the pWE probe (C). See legends for

35　explanation of sample loading. These experiments show that

all lanes that contain material from Cos-1 cells that are
transfected with plasmids harboring an SV40 ori contain *MboI*
sensitive DNA and show a specific band of the expected
length. The bands specific for replication in the lanes with
5   Cos-1 cells transfected with *PacI* digested material (lanes B
17/18 and C 15-18) probably result from incomplete *PacI*
digestion. From these experiments it can be concluded that it
is possible to replicate large DNA fragments with the SV40
LargeT/*ori* system in eukaryotic cells.

10   **Example 8**

        A functional adenovirus helper molecule lacking ITR
sequences was constructed starting with the clone pWE/Ad.D5'
described *supra*. pWE/Ad.D5' was digested with *Bstl107I* and
the 17.5 kb vector-containing fragment was religated to give
15   pWE/Ad.D5'-Bstl107I. This clone was then used to amplify the
3' part of the adenovirus genome sequences without the right
ITR. A 2645 bp PCR fragment was generated using the primers
Ad3'/Forw: 5'-CGG AAT TCA TCA GGA TAG GGC GGT GG-3' and
Ad3'/Rev: 5'-CGG GAT CCT ATC GAT ATT TAA ATG TTT TAG GGC GGA
20   GTA ACT TG-3'. The amplified fragment was digested with *EcoRI*
and *BamHI* and subcloned in pBr322 digested with the same
enzymes. After confirmation of correct amplification by
sequencing, the 2558 bp *SbfI-ClaI* fragment of this clone was
recloned in pWE/Ad.D5'-Bstl1071 digested with the same
25   enzymes. The resulting construct lacks the right ITR and is
named pWE/Δrl-Bstl107I. Next, in this clone the left ITR was
replaced by a linker with a *PacI* and *AflII* overhang made up
by annealing the following primers: PA-pIX1 5'-TAA GCC ACT
AGT ACG TAC TGA AAT GTG TGG GCG TGG C-3' and PA-pIX2 5'-TTA
30   AGC CAC GCC CAC ACA TTT CAG TAC GTA CTA GTG GCT TAAT-3'. This
removed the left ITR and restored correct sequence of the pIX
promoter. The clone is named pWE/ΔITRBstl107I. Correct
insertion of the double stranded linker was confirmed by
sequencing. The deleted Bstl107I fragment was then cloned

back into pWE/ΔITR-Bst1107I and the correct orientation was
checked by restriction digestion. The resulting clone is
named pWE/Ad-H. Following transfection of this DNA molecule
into packaging cells that express adenoviral E1 proteins and

5   the SV40 Large T antigen, replication of that molecule takes
place resulting in high levels of adenoviral proteins encoded
by the adenoviral entity on that molecule.

Example 9

10

Additional modifications of adapter plasmids

To enable removal of vector sequences from the left ITR in
pAd5/Clip (described in example 2B), this plasmid was

15   partially digested with EcoRI and the linear fragment was
isolated. An oligo of the sequence 5' TTAAGTCGAC-3' was
annealed to itself resulting in a linker with a SalI site and
EcoRI overhang. The linker was ligated to the partially
digested pAd5/Clip vector and clones were selected that had

20   the linker inserted in the EcoRI site 23 bp upstream of the
left adenovirus ITR in pAd5/Clip resulting in pAd5/Clipsal.
Likewise, the EcoRI site in pAd5/Clip has been changed to a
PacI site by insertion of a linker of the sequence 5'-
AATTGTCTTAATTAACCGCAATT-3' (as described in example 2). The

25   pAd5/Clip was partially digested with EcoRI, dephosphorylated
and ligated to the PacI linker with EcoRI overhang. The
ligation mixture was digested with PacI to remove
concatamers, isolated from agarose gel and religated. The
resulting vector was named pAd5/Clippac. These changes enable

30   more flexibility to liberate the left ITR from the plasmid
vector sequences.
The vector pAd5/L420-HSA was also modified to create a SalI
or PacI site upstream of the left ITR. Hereto pAd5/L420-HSA
was digested with EcoRI and ligated to the above described

35   PacI linker. The ligation mixture was digested with PacI and
religated after isolation of the linear DNA from agarose gel

to remove concatamerised linkers. This resulted in adapter
plasmid pAd5/L420-HSApac. This construct was used to generate
pAd5/L420-HSAsal as follows: pAd5/L420-HSApac was digested
with ScaI and BsrGI and the vector fragment was ligated to
5   the 0.3 kb fragment isolated after digestion of pAd5/Clipsal
with the same enzymes.

Generation of adapter plasmids AdMire and AdApt
To create an adapter plasmid that only contains a polylinker
10 sequence and no promoter or polyA sequences, pAd5/L420-HSApac
was digested with AvrII and BglII. The vector fragment was
ligated to a linker oligonucleotide digested with the same
restriction enzymes. The linker was made by annealing oligos
of the following sequence:
15 PLL-1: 5'- GCC ATC CCT AGG AAG CTT GGT ACC GGT GAA TTC GCT
AGC GTT AAC GGA TCC TCT AGA CGA GAT CTG G-3' and
PLL-2: 5'- CCA GAT CTC GTC TAG AGG ATC CGT TAA CGC TAG CGA
ATT CAC CGG TAC CAA GCT TCC TAG GGA TGG C-3'.
The annealed linkers were digested with AvrII and BglII and
20 separated from small ends by column purification (Qiaquick
nucleotide removal kit) according to manufacterers
recommendations. The linker was then ligated to the
AvrII/BglII digested pAd5/L420-HSApac fragment. A clone,
named AdMire, was selected that had the linker incorporated
25 and was sequenced to check the integrity of the insert.
Adapter plasmid AdMire enables easy insertion of complete
expression cassettes.
An adapter plasmid containing the human CMV promoter that
mediates high expression levels in human cells was
30 constructed as follows: pAd5/L420-HSApac was digested with
AvrII and 5' protruding ends were filled in using Klenow
enzyme. A second digestion with HindIII resulted in removal
of the L420 promoter sequences. The vector fragment was
isolated and ligated to a PCR fragment containing the CMV
35 promoter sequence. This PCR fragment was obtained after

amplification of CMV sequences from pCMVLacI (Stratagene)
with the following primers:
CMVplus: 5'-GATCGGTACCA<u>CTGCAG</u>TGGTCAATATTGGCCATTAGCC-3' and
CMVminA: 5'-GATC<u>AAGCTT</u>CCAATGCACCGTTCCCGGC-3'.

5 The PCR fragment was first digested with PstI (underlined in
CMVplus) after which the 3'-protruding ends were removed by
treatment with T4 DNA polymerase. Then the DNA was digested
with HindIII (underlined in CMVminA) and ligated into the
above described pAd5/L420-HSApac vector fragment digested

10 with AvrII and HindIII. The resulting plasmid was named
pAd5/CMV-HSApac. This plasmid was then digested with HindIII
and BamHI and the vector fragment was isolated and ligated to
the polylinker sequence obtained after digestion of AdMire
with HindIII and BglII. The resulting plasmid was named

15 AdApt. Adapter plasmid AdApt contains nucleotides -735 to +95
of the human CMV promoter (Boshart et al., 1985; M. Boshart,
F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein and W.
Schaffner. A very strong enhancer is located upstream of an
immediate early gene of human cytomegalovirus. Cell 41,521-

20 530, 1985). A second version of this adapter plasmid
containing a SalI site in place of the PacI site upstream of
the left ITR was made by inserting the 0.7 kb ScaI-BsrGI
fragment from pClipsal into AdApt digested with ScaI and
partially digested with BsrGI. This clone was named

25 AdApt.sal.

Example 10

<u>Modifications on adenoviral plasmids</u>

30

Generation of pWE/Ad.AflII-rITRΔE2A:
Deletion of the E2A coding sequences from pWE/Ad.AflII-rITR
(ECACC deposit P97082116) has been accomplished as follows.
The adenoviral sequences flanking the E2A coding region at

35 the left and the right site were amplified from the plasmid
pBr/Ad.Sal.rITR (ECACC deposit P97082119) in a PCR reaction

with the Expand PCR system (Boehringer) according to the
manufacturers protocol. The following primers were used:

Right flanking sequences (corresponding Ad5 nucleotides
24033 to 25180):

ΔE2A.SnaBI: 5'-GGC GTA CGT AGC CCT GTC GAA AG-3'

ΔE2A.DBP-start: 5'-CCA ATG CAT TCG AAG TAC TTC CTT
CTC CTA TAG GC-3'

The amplified DNA fragment was digested with SnaBI and NsiI
(NsiI site is generated in the primer ΔE2A.DBP-start,
underlined).

Left flanking sequences (corresponding Ad5 nucleotides
21557 to 22442):

ΔE2A.DBP-stop: 5'-CCA ATG CAT ACG GCG CAG ACG G-3'

ΔE2A.BamHI: 5'-GAG GTG GAT CCC ATG GAC GAG-3'

The amplified DNA was digested with BamHI and NsiI (NsiI site
is generated in the primer ΔE2A.DBP-stop, underlined).
Subsequently, the digested DNA fragments were ligated into
SnaBI/BamHI digested pBr/Ad.Sal-rITR. Sequencing confirmed
the exact replacement of the DBP coding region with a unique
NsiI site in plasmid pBr/Ad.Sal-rITRΔE2A. The unique NsiI
site can be used to introduce an expression cassette for a
gene to be transduced by the recombinant vector.

The deletion of the E2A coding sequences was performed
such that the splice acceptor sites of the 100K encoding L4-
gene at position 24048 in the top strand was left intact. In
addition, the poly adenylation signals of the origional E2A-
RNA and L3-RNAs at the left hand site of the E2A coding
sequences were left intact. This ensures proper expression of
the L3-genes and the gene encoding the 100K L4-protein during
the adenovirus life cycle.

Next, the plasmid pWE/Ad.AflII-rITRΔE2A was generated. The
plasmid pBr/Ad.Sal-rITRΔE2A was digested with BamHI and SpeI.
The 3.9-Kb fragment in which the E2A coding region was
replaced by the unique NsiI site was isolated. The
pWE/Ad.AflII-rITR was digested with BamHI and SpeI. The 35 Kb
DNA fragment, from which the BamHI/SpeI fragment containing

the E2A coding sequence was removed, was isolated. The
fragments were ligated and packaged using λ phage-packaging
extracts according to the manufacturer protocol (Stratagene),
yielding the plasmid pWE/Ad.AflII-rITRΔE2A.

5   This cosmid clone can be used to generate adenoviral vectors
that are deleted for E2A by cotransfection of PacI digested
DNA together with digested adapter plasmids onto packaging
cells that express functional E2A gene product. Examples of
E2A complementing cell lines are described infra.

10

Generation of pWE/Ad.AflII-rITRsp

The 3' ITR in the vector pWE/Ad.AflII-rITR does not include
the terminal G-nucleotide. Furthermore, the PacI site is
located almost 30 bp from the right ITR. Both these

15   characteristics may decrease the efficiency of virus
generation due to inefficient initiation of replication at
the 3' ITR. Note that during virus generation the left ITR in
the adapter plasmid is intact and enables replication of the
virus DNA after homologous recombination.

20   To improve the efficiency of initiation of replication at the
3' ITR, the pWE/Ad.AflII-rITR was modified as follows:
construct pBr/Ad.Bam-rITRpac#2 was first digested with PacI
and then partially digested with AvrII and the 17.8 kb
vector containing fragment was isolated and dephophorylated

25   using SAP enzyme (Boehrringer Mannheim). This fragment lacks
the adenosequences from nucleotide 35464 to the 3'ITR. Using
DNA from pWE/Ad.AflII-rITR as template and the primers
ITR-EPH:
5'-CGG AAT TCT TAA TTA AGT TAA CAT CAT CAA TAA TAT ACC-3' and

30   Ad101: 5'-TGA TTC ACA TCG GTC AGT GC-3'
a 630 bp PCR fragment was generated corresponding to the 3'
Ad5 sequences. This PCR fragment was subsequently cloned in
the vector pCR2.1 (Invitrogen) and clones containing the PCR
fragment were isolated and sequenced to check correct

35   amplification of the DNA. The PCR clone was then digested
with PacI and AvrII and the 0.5 kb adeno insert was ligated

to the PacI/ partial AvrII digested pBr/Ad.Bam-rITRpac#2
fragment generating pBr/Ad.Bam-rITRsp. Next this construct
was used to generate a cosmid clone (as described in example
2) that has an insert corresponding to the adenosequences

5   3534 to 35938. This clone was named pWE/AflII-rITRsp.

Generation of adenovirus template clones lacking DNA encoding
for fiber
Adenovirus infection is mediated by two capsid proteins fiber

10   and penton. Binding of the virus to the cells is achieved by
interaction of the protruding fiber protein with a receptor
on the cell surface. Internalisation then takes place after
interaction of the penton protein with integrins on the cell
surface. At least some adenovirus from subgroup C and B have

15   been shown to use a different receptor for cell binding and
therefor have different infection efficiencies on different
cell types. Thus it is possible to change the infection
spectrum of adenoviruses by changing the fiber in the capsid.
The fiber coding sequence of adenovirus serotype 5 is located

20   between nucleotides 31042 and 32787. To remove the adenovirus
serotype 5 DNA encoding fiber we started with construct
pBr/Ad.Bam-rITR. First a NdeI site was removed from this
construct. For this purpose, pBr322 plasmid DNA was digested
with NdeI after which protruding ends were filled using

25   Klenow enzym. This pBr322 plasmid was then re-ligated,
digested with NdeI and transformed into *E.coli* DH5α. The
obtained pBr/ΔNdeI plasmid was digested with ScaI and SalI
and the resulting 3198 bp vector fragment was ligated to the
15349 bp ScaI-SalI fragment derived from pBr/Ad.BamrITR,

30   resulting in plasmid pBr/Ad.Bam-rITRΔNdeI which hence
contained a unique NdeI site. Next a PCR was performed with
oligonucleotides
NY-up:
5'- CGA **CAT ATG** TAG <u>ATG CAT</u> TAG TTT GTG TTA TGT TTC AAC GTG-

35   3'
and

NY-down:

5'-GGA GAC CAC TGC CAT GTT-3'

During amplification, both a NdeI (bold face) and a NsiI
restriction site (underlined) were introduced to facilitate

5 cloning of the amplified fiber DNAs. Amplification consisted
of 25 cycles of each 45 sec. at 94°C, 1 min. at 60°C, and 45
sec. at 72°C. The PCR reaction contained 25 pmol of
oligonucleotides NY-up or NY-down, 2mM dNTP, PCR buffer with
1.5 mM MgCl$_2$, and 1 unit of Elongase heat stable polymerase

10 (Gibco, The Netherlands). One-tenth of the PCR product was
run on an agarose gel which demonstrated that the expected
DNA fragment of ± 2200 bp was amplified. This PCR fragment
was subsequently purified using Geneclean kit system (Bio101
Inc.). Then, both the construct pBr/Ad.Bam-rITRΔNdeI as well

15 as the PCR product were digested with restriction enzymes
NdeI and SbfI. The PCR fragment was subsequently cloned using
T4 ligase enzyme into the NdeI and SbfI digested pBr/Ad.Bam-
rITRΔNdeI, generating pBr/Ad.BamRΔFib.

This plasmid allows insertion of any PCR amplified fiber

20 sequence through the unique NdeI and NsiI sites that are
inserted in place of the removed fiber sequence. Viruses can
be generated by a double homologous recombination in
packaging cells described *infra* using an adapter plasmid,
construct pBr/Ad.AflII-EcoRI digested with PacI and EcoRI and

25 a pBr/Ad.BamRΔFib construct in which heterologous fiber
sequences have been inserted. To increase the efficiency of
virus generation, the construct pBr/Ad.BamRΔFib was modified
to generate a PacI site flanking the right ITR. Hereto,
pBr/Ad.BamRΔFib was digested with AvrII and the 5 kb

30 adenofragment was isolated and introduced into the vector
pBr/Ad.Bam-rITR.pac#8 (described in example 2) replacing the
corresponding AvrII fragment. The resulting construct was
named pBr/Ad.BamRΔFib.pac.

Once a heterologous fiber sequence is introduced in

35 pBr/Ad.BamRΔFib.pac, the fiber modified right hand adenovirus
clone may be introduced into a large cosmid clone as

described for pWE/Ad.AflII-rITR in example 2. Such a large
cosmid clone allows generation of adenovirus by only one
homologous recombination making the process extremely
efficient.

5

Generation of adenovirus clones lacking DNA encoding hexon

A major limitation for gene therapy approaches using
adenovirus type 5-based recombinant adenoviruses is the
presence of neutralising antibodies in human serum. As much

10   as 80-90% of individuals contain neutralising immunity to
Ad5. The majority of the neutralising antibodies is directed
to the hexon protein. Hexon proteins from different serotypes
show highly variable regions present in loops that are
predicted to be exposed at the outside of the virus

15   (Athappilly et al., 1994; J. Mol. Biol. 242, 430-455). Most
type specific epitopes have been mapped to these highly
variable regions (Toogood et al., 1989; J. Gen Virol. 70,
3203-3214). Thus replacement of (part of) the hexon
sequences with corresponding sequences from a different

20   serotype is an effective strategy to circumvent (pre-
existing) neutralising antibodies to Ad5. Hexon coding
sequences of adenovirus serotype 5 are located between
nucleotides 18841 and 21697.
To facilitate easy exchange of hexon coding sequences from

25   alternative adenovirus serotypes into the adenovirus serotype
5 backbone, first a shuttle vector was generated. This
subclone, coded pBr/Ad.Eco-PmeI, was generated by first
digesting plasmid pBr322 with EcoRI and EcoRV and inserting
the 14 kb PmeI-EcoRI fragment from pWE/Ad.AflII-Eco. In this

30   shuttle vector a deletion was made of a 1430 bp SanDI
fragment by digestion with SanDI and religation to give
pBr/Ad.Eco-PmeI ΔSanDI. The removed fragment contains unique
SpeI and MunI sites. From pBr/Ad.Eco-PmeIΔSanDI the
adenovirus serotype 5 DNA encoding hexon was deleted. Hereto,

35   the hexon flanking sequences were PCR amplified and linked
together thereby generating unique restriction sites

replacing the hexon coding region. For these PCR reactions
four different oligonucleotides were required: Δhex1-Δhex4.

Δhex1: 5'- CCT GGT GCT GCC AAC AGC-3'

Δhex2: 5'- CCG **GAT** CC<u>A CTA GT</u>G GAA AGC GGG CGC GCG-3'

Δhex3: 5'- CCG **GAT** CC<u>A ATT G</u>AG AAG CAA GCA ACA TCA ACA AC-3'

Δhex4: 5'- GAG AAG GGC ATG GAG GCT G-3'

The amplified DNA product of ± 1100 bp obtained with
oligonucleotides Δhex1 and Δhex2 was digested with BamHI and
FseI. The amplified DNA product of ± 1600 bp obtained with
oligonucleotides Δhex3 and Δhex4 was digested with BamHI and
SbfI. These digested PCR fragments were subsequently purified
from agarose gel and in a tri-part ligation reaction using T4
ligase enzyme linked to pBr/Ad.Eco-PmeI ΔSanDI digested with
FseI and SbfI. The resulting construct was coded pBr/Ad.Eco-
PmeΔHexon. This construct was sequenced in part to confirm
the correct nucleotide sequence and the presence of unique
restriction sites MunI and SpeI.

pBr/Ad.Eco-PmeΔHexon serves as a shuttle vector to introduce
heterologous hexon sequences amplified from virus DNA from
different serotypes using primers that introduce the unique
restriction sites MunI and SpeI at the 5' and 3' ends of the
hexon sequences respectively. The hexon modified sequences
are subsequently introduced in the construct pWE/Ad.AflII-
rITR by exchange of the AscI fragment generating
pWE/Ad.AflII-rITRHexXX where XX stands for the serotype used
to amplify hexon sequences.

Generation of adenoviral clones lacking DNA encoding penton

The adenovirus type 5 penton gene is located between
sequences 14156 and 15869. Penton base is the adenovirus
capsid protein that mediates internalisation of the virus
into the target cell. At least some serotypes (type C and B)
have been shown to achieve this by interaction of an RGD
sequence in penton with integrins on the cell surface.
However, type F adenoviruses do not have an RGD sequence and
for most viruses of the A and D group the penton sequence is

not known. Therefor, penton may be involved in target cell
specificity. Furthermore, as a capsid protein, the penton
protein is involved in the immunogenicity of the adenovirus.
Individuals, including patients that are candidates for gene
5 therapy approaches, may have pre-existing antibodies directed
to penton proteins in their serum. The replacement of Ad5
penton sequences with penton sequences from certain other
serotypes therefor, will affect infection specficity as well
as immunogenicity of the virus. To be able to introduce
10 heterologous penton sequences in Ad5 we made use of the
plasmid-based system described *infra*. First a shuttle vector
for penton sequences was made by insertion of the 7.2 kb
NheI-EcoRV fragment from construct pWE/Ad.AflII-EcoRI
(described in example 2) into pBr322 digested with the same
15 enzymes. The resulting vector was named pBr/XN. From this
plasmid Ad5 penton sequences were deleted and replaced by
unique restriction sites that are then used to introduce new
penton sequences from other serotypes. Hereto, the left
flanking sequences of penton in pBr/XN were PCR amplified
20 using the following primers:
DP5-F: 5'- CTG TTG CTG CTG CTA ATA GC-3' and
DP5-R: 5'- CGC GGA TCC TGT ACA ACT AAG GGG AAT ACA AG-3'
DP5-R has an BamHI site (underlined) for ligation to the
right flanking sequence and also introduces a unique BsrGI
25 site (bold face) at the 5'-end of the former Ad5 penton
region.
The right flanking sequence was amplified using:
DP3-F: 5'-CGC GGA TCC CTT AAG GCA AGC ATG TCC ATC CTT-3' and
DP3-3R: 5'- AAA ACA CGT TTT ACG CGT CGA CCT TTC-3'
30 DP3-F has an BamHI site (underlined) for ligation to the left
flanking sequence and also introduces a unique AflII site
(bold face) at the 3'-end of the former Ad5 penton region.
The two resulting PCR fragments were digested with BamHI and
ligated together. Then this ligation mixture was digested
35 with AvrII and BglII. pBr/XN was also digested with AvrII and
BglII and the vector fragment was ligated to the digested

ligated PCR fragments. The resulting clone was named pBr/Ad.Δ
penton. Penton coding sequences from serotypes other than Ad5
were PCR amplified such that the 5' and 3' ends contained the
BsrGI and AflII sites respectively. Introduction of these
5   heterologous penton sequences in pBr/Ad.Δpenton generates
constructs named pBr/Ad.pentonXX where XX represents the
number of the serotype corresponding to the serotype used to
amplify the inserted penton sequences. Subsequently the new
penton sequences were introduced in the pWE/Ad.AflII-rITR
10   construct by exchanging the common FseI fragment.
Importantly, in stead of pWE/Ad.AflII-rITR it is also
possible to insert the FseI fragment from pBr/Ad.pentonXX
into a pWE/Ad.AfllII-rITR vector having a modified hexon
and/or fiber sequence. In this way the plasmid-based system
15   to generate adenoviruses enables flexible design of any
adenovirus with any desired characteristic concerning
efficiency and specificity of infection of the target cell as
well as immunogenicity.

20   **Example 11**

Generation of replicating viruses
The plasmid-based system to generate recombinant adenoviruses
described *infra* is also very well suited to generate
25   replicating viruses. Replicating viruses may be used for gene
therapy approaches aimed at eradicating tumor cells. For
example suicide gene therapy methods using replicating
adenoviruses that express the Herpes simplex virus-1
thymidine kinase gene (HSV-tk) may have improved efficacy due
30   to increased spread of the vector. Safety is ensured by the
possibility to block replication at any time by
administration of ganciclovir.
Replicating viruses expressing HSV-tk or a marker gene have
been generated with the double homologous recombination
35   system described in example 2. Hereto, the following
constructs are transfected onto packaging cells:

- pBr/Ad.1ITR-SalI(9.4), digested with EcoRI and SalI to
  liberate the adeno insert from the vector sequences.
- pWE/Ad.AflII-EcoRI digested with PacI and EcoRI
- pBr/Ad.Bam-rITRΔgp19K/luc² or pBr/Ad.Bam-rITRΔgp19K/TK

5   digested with SalI,
whereby the third construct is a derivative of the
pBr/Ad.Bam-rITR construct that is made by replacement of the
gp19K coding region with either a marker gene (luciferase) or
the HSV-tk gene as described in example 2 for E1-deleted

10   viruses with modifications in the E3 region. In stead of
pBr/Ad.Bam-rITR, the modification of the E3 region may also
be introduced in pBr/Ad.Bam-rITRpac#2 or #8, or in
pBr/Ad.Bam-rITRsp. This enables liberation of the right ITR
from the vector sequences by digestion with PacI and

15   increases the efficiency by which viruses are generated.

Example 12

Generation of recombinant viruses using the described
20   *plasmid-based system is extremely efficient and reliable*

Several methods have been described previously for the
generation of recombinant adenoviruses. One of these methods
makes use of a circular large adenoviral plasmid that is

25   cotransfected on packaging cells with a linearised adapter
plasmid (Bett *et al.*,1994). The efficiency of this method is
low due to the fact that the ITRs are linked head to head in
the large adenoviral plasmid. Other methods make use of a
recombination step in specialised bacteria that lead to a

30   recombinant viral DNA clone (Chartier *et al.*, 1996; Crouzet
et al., 1997; He *et al.*, 1998). After restriction analysis of
the clones and selection of correct recombinants a different
strain of bacteria has to be transformed to make a large
batch of the DNA. Then linearised fragments are transfected

35   in packaging cells and recombinant viruses appear within a
week following transfection.

The plasmid system described *infra* differs from the methods
described above. The system combines easy manipulation of
small adapter plasmids in standard bacteria with efficient
homologous recombination in packaging cells due to linearised
5   large adenoviral plasmids.
The high efficiency of homologous recombination in E1-
complementing packaging cells is examplified by the
experiment described below.
A 96-well microtiter tissue culture plate (plate 1) (Greiner,
10   The Netherlands, catalogue #6555180) was first coated with
poly-L-lysine (PLL, 0.1 mg/ml) (Sigma) dissolved in sterile
water by incubating each well for 20-120 minutes at room
temperature. Alternatively, precoated 96-well plates can be
used (Becton and Dickinson). After the incubation with PLL,
15   each well was washed two times with 100 μl sterile water and
dried at room temperature for at least two hours. The day
before transfection PER.C6 cells were harvested using
trypsin-EDTA and counted. The cells were then diluted to a
suspension of 45,000 cells per 100 μl followed by seeding 100
20   μl per well of the PLL coated 96-well plates. The next day
2.6 μl of *Sal* I linearized pAd/CMV-LacZ and 2.6 μl of *Pac*I
linearized pWE-Ad.AflII-rITR plasmid DNA (both 1 μg/μl) and
95 μl serum free Dulbecco's Modified Eagles Medium (DMEM)
were mixed with 25.6 μl lipofectamine diluted in 74.4 μl
25   serum free DMEM by adding the lipofectamine to the DNA mix.
The DNA/lipofectamine mixture was left at room temperature
for 30 minutes after which 1.3 ml serum free media was added.
The latter mixture was then added (30 μl per well) to PER.C6
seeded wells that were washed with 200 μl DMEM prior to
30   transfection. After 3 hours in a humidified CO_2 incubator
(37°C, 10% CO_2) 200 μl DMEM with 10% fetal calf serum 10 mM
$MgCl_2$ was added to each well and the plates were returned to
the humidified CO_2 incubator (37°C, 10% CO_2). The next day
the medium of each well was replaced with 200 μl DMEM,
35   10% FCS, 10 mM $MgCl_2$. The plates were then left in the
humidified CO_2 incubator for an additional three days after

which the wells were subjected to freezing at -20°C for at least 1 hour followed by thawing and resuspension by repeated pipetting. Transfection efficiency was determined using lacZ staining in additional plates and found to be approximately

5   40% for each transfected well of PER.C6 cells. An aliquot of 100 µl of freeze/thawed transfected cells was transferred to each well of a plate with new PER.C6 cells seeded as described above without PLL coated plates (plate 2). The second 96-well plate with PER.C6 cells incubated with

10   freeze/thaw cell lysate of the first transfected plate was checked for CPE. At least 5% of the wells showed clear CPE after 2 days. Four days after infection with the lysate from plate 1 the plate was subjected to one freeze-thaw cycle and 10 µl from each lysed well was added to wells of a plate

15   seeded with A549 cells (1 x 10⁴ cells per well seeded in 100 µl in DMEM, 10% FCS the day before). Two days after infection the wells were stained for lacZ activity. Of the infected wells 96% were infected and stained blue. All wells stained and a large number of wells showed 100% blue staining

20   and thus transduction of all cells with adenoviral vector carrying lacZ. Extrapolated from MOI experiments in tissue culture flasks the adenoviral titer of well-produced virus is around 10⁶-10⁷ infectious units per ml.

From the high percentage of wells that contain LacZ virus we

25   conclude that the plasmid-based system for the generation of adenoviruses described *infra* is very efficient.

In addition to highly efficient, the system is also very reliable. Using the routine procedure of virus generation as described in example 2 (section C) we obtain a T80 flask with

30   adenovirus infected cells showing full CPE. Different stocks of viruses obtained after cotransfection with adapter plasmids carrying different transgenes (Luciferase, LacZ, ratIL-3, humanIL1α, HSV1-TK, ceNOS, hgp100) and different promoters (MLP, CMV, E3 or retroviral LTR) in a total of 16

35   transfections, were subjected to plaque purification and separate plaques were tested for expression of the transgene.

Out of a total of 145 plaques only two were found to be
negative. When positive plaques from the first plaque
purification were subjected to a second round of plaque
purification all tested plaques were found to be positive
5   (144 out of 144 tested). This clearly shows that the plasmid-
based system of the invention is very reliable.

Example 13

10   *Generation of minimal adenoviral vectors with large inserts*

In examples 7 and 8 methods are described for the production
of minimal adenoviral vectors in E1 expressing packaging
cells. The minimal vectors described here only contain an
15   expression cassette for a gene of interest and the adenovirus
ITRs and packaging sequences. Efficient packaging of
adenoviruses requires a genome length of >27 kb (Parks and
Graham, 1997; J.Virol. 71, 3293-3298). Therefor, to be able
to produce high titers of minimal adenoviruses it is
20   necessary to include stuffer DNA in the vectors to reach the
optimal packaging size. In case one designs a gene correction
vector it is possible or even necessary to include a large
fragment of genomic DNA homologous to the genome site to be
targetted. In other cases the gene of interest may not be
25   large enough to fulfill the packaging size and a stuffer has
to be included. Here we describe the construction of larger
minimal vectors with stuffer DNA and a method to produce such
vectors.
The vectors pMV/L420H (figure 24) and pMV/CMV-LacZ were first
30   modified to create a second NotI site flanking the left ITR.
Hereto, pMV/L420H was partially digested with EcoRI and the
linear fragment was isolated. This fragment was ligated to a
double stranded linker obtained by annealing an
oligonucleotide of the sequence 5'-AATTGCGGCCGC-3'. A clone
35   was selected which had the NotI linker inserted in the
correct EcoRI site. This clone was named pMV/L420H.nn. Next

pMV/CMV-LacZ was digested with ScaI and BsrGI and the 7 kbp
fragment lacking part of the Amp gene and adenovirus ITR was
isolated. This fragment was then ligated to the 0.7 kbp ScaI-
BsrGI fragment from pMV/L420H.nn. This resulted in pMV/CMV-

5   LacZ.nn. In both minimal vectors pMV/L420H.nn and pMV/CMV-
LacZ.nn, the ITRs are flanked by a NotI site. Since the
vector backbone is a cosmid vector based on pWE15 these
clones can be used to insert large fragments of stuffer DNA.
Stuffer inserts can be any piece of DNA that contains no

10 active transcription regions. Alternatively, the described
minimal vectors can be used to insert a large fragement of
genomic DNA. In case a marker gene is not required, the
insertion can be such that the expression cassette for the
HSA gene or the LacZ gene is replaced by the genomic fragment

15 by making use of the SnaBI of AvrII site at the 5' end of the
expression cassette and the unique sites at the 3' end.
One example of a suitable stuffer DNA is a part of the 44th
intron of the human Dysthrophin genomic DNA (Genbank
accession code: M86524). The generation of large cosmid

20 clones containing the above described minimal adenoviral
vectors and 31.7 kb of the dystrophin intron sequence are
described below. Hereto the dystrophin sequence is digested
with XhoI and BstBI and the 31.7 kb fragments is isolated.
Part of the fragment is left with sticky ends and part is

25 filled in with Klenow. Then pMV/L420H.nn is digested with
XhoI and ClaI and ligated to the dystrophin fragment with
sticky ends. pMV/CMV-LacZ.nn is digested with XhoI, blunted
with Klenow enzyme and ligated to the blunted dystrophin
fragment. Both ligations are packaged as described before.

30 The large clones generated in this way tend to be unstable in
bacteria probably due to the large insert and the presence of
two inverted terminal repeats of adenovirus. An improved
method to generate these large minimal vectors is described
below. This method makes use of the powerfull system of

35 homologous recombination in packaging cells for the
generation of recombinant viruses.

Generation of minimal adenoviral vectors by homologous
recombination in packaging cells.

pMV/CMV-LacZ.nn was digested with XhoI and NsiI and the ends
were blunted with T4 DNA polymerase. The linear fragment is
5   isolated and ligated to the approximately 17.5 kbp XhoI/KpnI
fragment from the dystrophin intron also blunted with T4 DNA
polymerase. Clones were selected that contained the
dystrophin fragment in the 5' to 3' direction. This clone,
named pMV/CMV-LacZ.Dys5' contains the left ITR and packaging
10 signal of adenovirus in addition to the LacZ expression
cassette and the 5' part of the dystrophin insert. A second
clone was then made by digestion of pMV/CMV-LacZ.nn with
BglII. The linear fragment was then partially digested with
NotI and the 6.4 kb vector fragment was blunted with Klenow
15 and isolated. This fragment was then ligated to the 18.8 kb
PvuI-BstBI dystrophin fragment blunted with T4 DNA
polymerase. Clones were selected that contained the
dystrophin fragment in the 5' to 3' direction. This clone,
named pMV/Dys3'-ITR contains a dystrophin insert that has 4.5
20 kb overlap with the dystrophin fragment in pMV/CMV-
LacZ.Dys5'.
Minimal adenoviral vectors containing the full length 31.7 kb
XhoI-BstBI fragment are generated by cotransfection of
adenovirus packaging cells (e.g. PER.C6) with pMV/CMV-
25 LacZ.Dys5' and pMV/Dys3' with an adenoviral helper plasmid as
described in this invention, for example pWE/Ad.D5' or
pWE/AdH and a SV40 LargeT expression construct (see examples
7 and 8). Hereto, the pMV/CMV-LacZ.Dys5' construct is
digested with NotI and BstBI and the pMV/Dys3' is digested
30 with NotI and PvuI to liberate the ITRs from the vector
sequences and to minimize the amount of vector DNA attached
to the dystrophin insert thereby enabling homologous
recombination and replication.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Construction of pBS.PGK.PCRI. pBS.PGK.PCRI encodes the human phosphoglycerate kinase promoter (PGK) operatively linked to adenovirus 5 (Ad5)E1 nucleotides 459-916. To construct this plasmid, Ad5 nucleotides 459-916 were PCR amplified with primers Ea-1 and Ea-2, digested with Cla I and cloned into the Cla I-EcoR V sites of pBluescript (Stratagene), resulting in pBS.PCRI. The PGK promoter was excised from pTN by complete digestion with Sca I and partial digestion with EcoR I and cloned into the corresponding sites of pBS.PCRI, resulting in pBS.PGK.PCRI.

Figure 2. Construction of pIG.E1A.E1B.X.
pIG.E1A.E1B.X encodes Ad5 nucleotides 459-5788 (E1A and E1B regions) operatively linked to the human PGK promoter. pIG.E1A.E1B.X also encodes Ad5 pIX protein. pIG.E1A.E1B.X was constructed by replacing the Sca I-BspE I fragment of pAT-X/S with the corresponding fragment of pBS.PGK.PCRI.

Figure 3. Construction of pIG.E1A.NEO.pIG.E1A.NEO encodes Ad5 nucleotides 459-1713 operatively linked to the human PGK promoter. Also encoded is the E1B promoter functionally linked to the neomycin resistance gene (Neo^R) and the hepatitis B virus (HBV) poly(A) signal. In this construct, the AUG codon of the E1B 21 kDa protein functions as the initiation codon of Neo^R. To construct this plasmid, the E1B promoter and initiation codon (ATG) of the E1B 21 kDa protein were PCR amplified with primers Ea-3 and Ep-2, where Ep-2 introduces an Nco I site (5'-CCATGG) at the 21 kDa protein initiation codon. The PCR product (PCII) was digested with Hpa I and Nco I and ligated into the corresponding sites of pAT-X/S, producing pAT-X/S-PCR2. The Nco I-Stu I fragment of pTN, containing the Neo^R and a portion of the HBV poly(A) site were ligated into the Nco I-Nru I sites of pAT-X/S-PCR2, producing pAT-PCR2-NEO. The HBV poly(A) signal was completed

by replacing the *Sca* I-*Sal* I fragment of pAT-PCR2-NEO with
the corresponding fragment of pTN, producing
pAT.PCR2.NEO.p(A), and replacing the *Sca* I-*Xba* I fragment of
pAT.PCR2.NEO.p(A) with the corresponding fragment of
5 pIG.E1A.E1B.X, producing pIG.E1A.NEO.

   Figure 4. Construction of pIG.E1A.E1B. pIG.E1A.E1B
contains the Ad5 nucleotides 459-3510 (E1A and E1B proteins)
operatively linked to the PGK promoter and HBV poly(A)
signal. This plasmid was constructed by PCR amplification of
10 the N-terminal amino acids of the E1B 55 kD protein with
primers Eb-1 and Eb-2, which introduces an *Xho* I site,
digested with *Bgl* II and cloned into the *Bgl* II-*Nru* I sites
of pAT-X/S, producing pAT-PCR3. The *Xba* I-*Xho* I fragment of
pAT-PCR3 was replaced with the *Xba* I-*Sal* I fragment
15 (containing the HBV poly(A) site) of pIG.E1A.NEO to produce
pIG.E1A.E1B.

   Figure 5. Construction of pIG.NEO. pIG.NEO contains
the NEO^R operatively linked to the E1B promoter. pIG.NEO was
constructed by ligating the *Hpa* I-*Sca* I fragment of
20 pIG.E1A.NEO which contains the E1B promoter and Neo^R into the
EcoR V-*Sca* I sites of pBS.

   Figure 6. Transformation of primary baby rat kidney
(BRK) cells by adenovirus packaging constructs. Subconfluent
dishes of BRK cells were transfected with 1 or 5 µg of with
25 either pIG.NEO, pIG.E1A.NEO, pIG.E1A.E1B, pIG.E1A.E1B.X,
pAd5XhoIC, or pIG.E1A.NEO plus pDC26, which expresses the Ad5
E1B gene under control of the SV40 early promoter. Three
weeks post-transfection, foci were visible, cells were fixed,
Giemsa stained and the foci counted. The results shown are
30 the average number of foci per 5 replicate dishes.

   Figure 7. Western blot analysis of A549 clones
transfected with pIG.E1A.NEO and human embryonic retinoblasts
(HER cells) transfected with pIG.E1A.E1B (PER clones).
Expression of Ad5 E1A and E1B 55 kD and 21 kD proteins in
35 transfected A549 cells and PER cells was determined by

Western blot with mouse monoclonal antibodies (Mab) M73 which recognizes E1A gene products and Mabs AIC6 and C1G11, which recognize the E1B 55 kDa and 21 kDa proteins, respectively. Mab binding was visualized using horseradish peroxidase-
5 labeled goat anti-mouse antibody and enhanced chemiluminesence. 293 and 911 cells served as controls.

Figure 8. Southern blot analysis of 293, 911 and PER cell lines. Cellular DNA was extracted, *Hind* III digested, electrophoresed and transferred to Hybond N'membranes
10 (Amersham). Membranes were hybridized to radiolabeled probes generated by random priming of the *Ssp* I-*Hind* III fragment of pAd5.SalB (Ad5 nucleotides 342-2805).

Figure 9. Transfection efficiency of PER.C3, PER.C5, PER.C6 and 911 cells. Cells were cultured in 6-well plates
15 and transfected in duplicate with 5 μg pRSV.lacZ by calcium-phosphate co-precipitation. Forty-eight hours post-transfection, cells were stained with X-Gal and blue cells were counted. Results shown are the mean percentage of blue cells per well.

20 Figure 10. Construction of adenovirus vector, pMLPI.TK. pMLPI.TK was designed to have no sequence overlap with the packaging construct pIG.E1A.E1B. pMLPI.TK was derived from pMLP.TK by deletion of the region of sequence overlap with pIG.E1A.E1B and deletion of non-coding sequences
25 derived from LacZ. SV40 poly(A) sequences of pMLP.TK were PCR amplified with primers SV40-1, which introduces a *BamH* I site and SV40-2, which introduces a *Bgl* II site. pMLP.TK Ad5 sequences 2496 to 2779 were PCR amplified with primers Ad5-1, which introduces a *Bgl* II site and Ad5-2. Both PCR products
30 were *Bgl* II digested, ligated, and PCR amplified with primers SV40-1 and Ad5-2. This third PCR product was *BamH* I and *Afl* III digested and ligated into the corresponding sites of pMLP.TK, producing PMLPI.TK.

Figure 11A-B. New adenovirus packaging constructs do
35 not have sequence overlap with new adenovirus vectors.

Regions of sequence overlap between the packaging construct,
pAd5XhoIC expressed in 911 cells and adenovirus vector,
pMLP.TK, that can result in homologous recombination and the
formation of replication competent adenovirus are shown
5   (Panel A). In contrast, there are no regions of sequence
overlap between the new packaging construct, pIG.E1A.E1B,
expressed in PER.C6 cells, and the new adenovirus vector,
pMLPI.TK, (Panel A) or between the new packaging construct,
pIG.E1A.NEO and the new adenovirus vector pMLPI.TK (Panel B)
10   that can result in homologous recombination and the formation
of replication competent adenovirus.

          Figure 12. Generation of recombinant adenovirus,
IG.Ad.MLPI.TK. Recombinant adenovirus, IG.Ad.MLPI.TK, was
generated by co-transfection of 293 cells, with *Sal* I
15   linearized pMLPI.TK and the right arm of Cla I digested,
wild-type Ad5 DNA. Homologous recombination between
linearized pMLPI.TK and wild-type Ad5 DNA produces
IG.Ad.MLPI.TK DNA, which contains an E1 deletion of
nucleotides 459-3510. 293 cells transcomplement the deleted
20   Ad5 genome, thereby, permitting replication of the
IG.Ad.MLPI.TK DNA and its packaging into virus particles.

          Figure 13. Rationale for the design of adenovirus-
derived recombinant DNA molecules that duplicate and
replicate in cells expressing adenovirus replication
25   proteins. A diagram of the adenovirus double-stranded DNA
genome indicating the approximate locations of E1, E2, E3,
E4, and L regions is shown. The terminal polypeptide (TP)
attached to the 5'-termini is indicated by closed circles.
The right arm of the adenovirus genome can be purified by
30   removal of the left arm by restriction enzyme digestion.
Following transfection of the right arm into 293 or 911
cells, adenoviral DNA polymerase (white arrow) encoded on the
right arm, will produce only single-stranded forms. Neither
the double-stranded or single-stranded DNA can replicate
35   because they lack an ITR at one termini. Providing the
single-stranded DNA with a sequence that can form a hairpin

structure at the 3'-terminus that can-serve as a substrate
for DNA polymerase will extend the hairpin structure along
the entire length of the molecule. This molecule can also
serve as a substrate for a DNA polymerase but the product is
5   a duplicated molecule with ITRs at both termini that can
replicate in the presence of adenoviral proteins.

Figure 14. Adenovirus genome replication. The
adenovirus genome is shown in the top left. The origins of
replication are located within the left and right ITRs at the
10   genome ends. DNA replication occurs in two stages.
Replication proceeds from one ITR generating a daughter
duplex and a displaced parental single-strand which is coated
with adenovirus DNA binding protein (DBP, open circles) and
can form a panhandle structure by annealing of the ITR
15   sequences at both termini. The panhandle is a substrate for
DNA polymerase (Pol: white arrows) to produce double-stranded
genomic DNA. Alternatively, replication proceeds from both
ITRs, generating two daughter molecules, thereby, obviating
the requirement for a panhandle structure.

20   Figure 15. Potential hairpin conformation of a
single-stranded DNA molecule that contains the HP/asp
sequence. Asp718 I digestion of pICLha, containing the cloned
oligonucleotides, HP/asp1 and HP/asp2 yields a linear double-
stranded DNA with an Ad5 ITR at one terminus and the HP/asp
25   sequence at the other terminus. In cells, expressing the
adenovirus E2 region, a single-stranded DNA is produced with
an Ad5 ITR at the 5'-terminus and the hairpin conformation at
the 3'-terminus. Once formed, the hairpin can serve as a
primer for cellular and/or adenovirus DNA polymerase to
30   convert the single stranded DNA to double stranded DNA.

Figure 16. Diagram of pICLhac. pICLhac contains all
the elements of pICL (Figure 19) but also contains in the
Asp718 site, the HP/asp sequence in an orientation that will
produce the hairpin structure shown in Figure 15, following
35   linearization by Asp718 digestion and transfection into cells
expressing adenovirus E2 proteins.

Figure 17. Diagram of pICLhaw. pICLhaw is identical
to pICLhac (Figure 16) with the exception that the inserted
HP/asp sequence is in the opposite orientation.

Figure 18. Schematic representation of pICLI. pICLI
5 contains all the elements of pICL (Figure 19) but also
contains in the Asp718 site, an Ad5 ITR.

Figure 19. Diagram of pICL. pICL is derived from the
following: (i) nucleotides 1-457, Ad5 nucleotides 1-457
including the left ITR, (ii) nucleotides 458-969, human CMV
10 enhancer and immediate early promoter, (iii) nucleotides 970-
1204, SV40 19S exon and truncated 16/19S intron, (iv)
nucleotides 1218-2987, firefly luciferase gene, (v)
nucleotides 3018-3131, SV40 tandem polyadenylation signals
from the late transcript, (vi) nucleotides 3132-5620, pUC12
15 sequences including an Asp718 site, and (vii) ampicillin
resistance gene in reverse orientation.

Figure 20: Shows a schematic overview of the
adenovirus fragments cloned in pBr322 (plasmid) or pWE15
(cosmid) derived vectors. The top line depicts the complete
20 adenovirus genome flanked by its ITRs (filled rectangles) and
with some restriction sites indicated. Numbers following
restriction sites indicate approximate digestion sites (in
kb) in the Ad5 genome.

Figure 21: Drawing of adapter plasmid pAd/L420-HSA
25   Figure 22: Drawing of adapter plasmid pAd/Clip
Figure 23: Schematic presentation of the generation
of recombinant adenoviruses using a plasmid-based system. In
the top the genome organization of Ad5 is given with filled
boxes representing the different early and late transcription
30 regions, and flanking ITRs. The middle presents the two DNAs
used for a single homologous recombination and, after
transfection into packaging cells, leading to the recombinant
virus (represented at the bottom).

Figure 24: Drawing of minimal adenoviral vector
35 pMY/L420H

Figure 25: Schematic presentation of the cloning
steps for the generation of the helper construct PWE/AdΔ5'

Figure 26: Evidence for SV40-LargeT/ori mediated
replication of large adenoviral constructs in Cos-1 cells. A)
5   Schematic presentation of construct pWE/Ad.Δ5' and the
location of the SV40 ori sequence and the fragments used to
prepare probes. B) Autoradiogram of the Southern blot
hybridized to the adenovirus probe. C) Autoradiogram of the
Southern blot hybridized to the pWE probe. Lanes 1, marker
10   lane: λ DNA digested with EcoRI and HindIII. Lane 4 is empty.
Lanes 2, 5, 7, 9, 11, 13, 15 and 17 contain undigested DNA
and Lanes 3, 6, 8, 10, 12, 14, 16 and 18 contain MboI
digested DNA. All lanes contain DNA from Cos-1 cells as
described in the text transfected with pWE.pac (lanes 2 and
15   3), pWE/Ad.Δ5' construct #1 (lanes 5 and 6), #5 (lanes 7 and
8) and #9 (lanes 9 and 10), pWE/Ad.AfIII-rITR (lanes 11 and
12), pMV/CMV-LacZ (lanes 13 and 14), pWE.pac digested with
PacI (lanes 15 and 16) or pWE/Ad.AfIII-rITR digested with
PacI (lanes 17 and 18). Arrows point at the expected positive
20   signal of 1416 bp(B) and 887 bp (C).

All publications and patent applications mentioned in
this specification are indicative of the level of skill of
25   those skilled in the art to which this invention pertains.
All publications and patent applications are herein
incorporated by reference to the same extent as if each
individual publication or patent application was specifically
and individually indicated to be incorporated by reference.
30   The invention now having been fully described, it
will be apparent to one of ordinary skill in the art that
many changes and modifications can be made thereto without
departing from the spirit or scope of the appended claims.

TABLE I

Primers used for PCR amplification of DNA fragments used for generation of constructs described in this patent application

Primer	Sequence	Description
Ea-1	CGTGTAGTGTATTTATACCCG	PCR amplification Ad5 nt.459 →
Ea-2	TCGTCACTGGGTGGAAAGCCA	PCR amplification Ad5 nt.960 ←
Ea-3	TACCCGCCGTCCTAAAATGGC	nt. 1284-1304 of Ad5 genome
Ea-5	TGGACTTGAGCTGTAAACGC	nt. 1514-1533 of Ad5 genome
Ep-2	GCCTCCATGGAGGTCAGATGT	nt. 1721-1702 of Ad5; introduction of NcoI site
Eb-1	GCTTGAGCCCGAGACACATGTC	nt. 3269-3289 of Ad5 genome
Eb-2	CCCCTCGAGCTCAATCTGTATCTT	nt. 3508-3496 of Ad5 genome; introduction of XhoI site
SV40-1	GGGGGATCCGAACTTGTTTATTGCAGC	introduction BamHI site (nt.2182-2199 of pMLP.TK) adaptation of recombinant adenoviruses
SV40-2	GGGAGATCTAGACATGATAAGATAC	introduction BglII site (nt.2312-2297 of pMLP.TK)
Ad5-1	GGGAGATCTGTACTGAAATGTGTGGGC	introduction BglII site (nt. 2496-2514 of pMLP.TK)
Ad5-2	GGAGGCTGCAGTCTCCAACGGCGT	nt.2779-2756 of pMLP.TK
ITR1	GGGGGATCCTCAAATCGTCACTTCCGT	nt.35737-35757 of Ad5 (introduction of BamHI site)
ITR2	GGGGTCTAGACACATCATCATCAATAATATAC	nt.35935-35919 of Ad5 (introduction of XbaI site)

TABLE I (Cont.)

PCR primer sets to be used to create the SalI and Asp718 sites juxtaposed to the ITR sequences.

PCR/MLP1   GGCGAATTCGTCGACACATCATCAATAATATACC   (Ad5 nt.10-18)

PCR/MLP2   GGCGAATTCGGTACCATCATCAATAATATACC   (Ad5 nt.10-18)

PCR/MLP3   CTGTGTACACCGGCGCA   (Ad5 nt.200-184)

Synthetic oligonucleotide pair used to generate a synthetic hairpin, recreates an Asp718 site at one of the termini if inserted in Asp718 site:

HP/asp1   5'-GTACACTGACCTAGTGCCGCCCGGGAAAGCCCGGGCGGCACTAGGTCAG

HP/asp2   5'-GTACCTGACCTAGTGCCGCCTAGTGCCCGGGCGGCACTAGGTCAGT

Synthetic oligonucleotide pair used to generate a synthetic hairpin, contains the ClaI recognition site to be used for hairpin formation.

HP/cla1   5'-GTACATTGACCTAGTGCCGCCCGGGCAAAGCCCGGGCGGCACTAGGTCAATCGAT

HP/cla2   5'-GTACATCGATTGACCTAGTGCCGCCCGGGTTTGCCCGGGCGGCACTAGGTCAAT

TABLE II

Production of recombinant adenoviral vector
or different packages, cell lines

Cell	Passage Number	IGAd.CMV.lacZ	IG.Ad.CMV.TK	IG.Ad.MLPI.TK	dl313	Producer Mean
		Yields x 10^{-8} pfu/T175 flask.[1]				
293		6.0	5.9	24	34	17.5
911		8	14	34	180	59.5
PER.C3	17	8	11	44	40	25.8
PER.C5	15	6	17	36	200	64.7
PER.C6	36	10	22	58	320	102

NOTE:
The yields are the mean of two different experiments. IG.Ad.CMV.lacz and IG.Ad.CMV.TK are described in patent application EP 95 20 2213. The construction of IG.Ad.MLPI.TK is described in this patent application. Yields of virus per T80 flask were determined by plaque assay on 911 cells, as described (Fallaux et al (1996) *Hum. Gene.Ther.* 7: 215-222). #1493).

TABLE III

Double insert viruses with different transgenes replacing the
E1 and E3/gp19K regions express both transgenes in human A549 cells

Virus[1]	Amount	% of cells with HSA expression	Luciferase activity (light units)
IGAd/CMV-Luc	5 x 10^7 i.u.	ND	25,726,074
	2.5 x 10^7 i.u.	ND	7,996,542
IGAd/S1800-HSA	100 µl ccl	88%	ND
	50 µl ccl	82%	ND
IGAd/S1800-HAS.E31luc	1.2 x 10^7 i.u.	97%	32,451,300
	6 x 10^7 i.u.	97%	24,716,586
	1.2 x 10^8 i.u.	100%	13,294,321

NOTE:

All virus preps were clarified crude cell lysates (ccl).

A clarified crude cell lysate was made by harvesting cells
with medium at full CPE followed by three freeze/thaw cycles.
pAd/S1800-HSA was not titrated.

Claims

1. A method for generating an adenovirus vector comprising welding together two nucleic acid molecules whereby said molecules comprise partially overlapping sequences capable of combining with each other allowing the

5 generation of a physically linked nucleic acid comprising at least two functional adenovirus inverted terminal repeats, a functional encapsulation signal and a nucleic acid of interest or functional parts, derivatives and/or analogues thereof.

10 2. A method for generating an adenovirus vector comprising welding together through homologous recombination two nucleic acid molecules comprising partially overlapping sequences wherein said overlapping sequences allow essentially only one homologous recombination which leads to

15 the generation of a physically linked nucleic acid comprising at least two functional adenovirus inverted terminal repeats, a functional encapsulation signal and a nucleic acid of interest or functional parts, derivatives and/or analogues thereof.

20 3. A method according to claim 1 or claim 2, wherein both of said nucleic acid molecules comprise only one adenovirus inverted terminal repeat or a functional part, derivative and/or analogue thereof.

4. A method according to anyone of claims 1-3, wherein

25 said welding together is performed in a cell or a functional part, derivative and/or analogue thereof.

5. A method according to claim 4, wherein said cell is a mammalian cell.

6. A method according to claim 5, wherein said nucleic

30 acid molecules are not capable of replicating in said mammalian cell prior to said welding together.

7. A method according to anyone of claims 1-6, wherein one of said nucleic acid molecules is relatively small and the other is relatively large.

8.   A method according to anyone of claims 1-7, wherein
at least one of said nucleic acid molecules provided to said
cell comprises an adenovirus inverted terminal repeat which
on one side is essentially free of other nucleic acid.

5   9.   A method according to claim 8, wherein said
adenovirus inverted terminal repeat is made essentially free
of other nucleic acid on one side using a restriction enzyme.
10.   A method according to claim 9, wherein said
restriction enzyme acts on a site which is not present in
10 adenovirus vector nucleic acid in said nucleic acid molecule.
11.   A method according to anyone of claims 4-10, wherein
the nucleic acids present in said cell do not comprise
sequence overlap that can lead to the formation of
replication competent adenovirus.
15 12.   A method according to anyone of claims 4-11, wherein
the chromosomal nucleic acid in said cell comprises at least
a functional part of an adenovirus E1-region, or a functional
derivative and/or analogue thereof.
13.   A method according to anyone of claims 4-12, wherein
20 said cell is a PER.C6 cell (ECACC deposit number 96022940) or
a functional derivative and/or analogue thereof.
14.   A method according to anyone of claims 4-13, wherein
said nucleic acid in said cell further comprises a nucleic
acid encoding an adenovirus E2-region and/or an adenovirus
25 E4-region protein.
15.   A method according to anyone of claims 1-14, wherein
at least one of said nucleic acid molecules is linear.
16.   A method according to anyone of claims 1-15, wherein
at least one of said molecules comprises adenovirus capsid
30 protein encoding nucleic acid derived from two different
adenovirus serotypes.
17.   A method according to anyone of claims 1-16, wherein
said welding together of said nucleic acid molecules leads to
the generation of a physically linked nucleic acid comprising
35 at least two functional adenovirus inverted terminal repeats,
a functional encapsulation signal, a nucleic acid encoding at

least one adenovirus E1-region protein, at least one adenovirus E2-region encoded protein and/or at least one adenovirus E4-region encoded protein and a nucleic acid of interest or functional parts, derivatives and/or analogues

5   thereof and wherein at least one of said E1-region encoded proteins is under transcriptional control of a conditionally active promoter.

18.   A recombinant nucleic acid deposited at the ECACC under No. P97082122, No. P97082119, No. P97082117,

10   No. P97082114, No. P97082120, No. P97082121, No. P97082116, No. P97082115 or No. P97082118 or a functional part, derivative and/or analogue thereof.

19.   A recombinant nucleic acid pWE/Ad.AflII-EcoRI, pAd5/CLIP, pAd5/L420-HAS, pBS.Eco-Eco/ad5ΔHIIIΔgp19KΔXbaI,

15   pMV/L420-H, pMV/CMV-LacZ, pWE/Ad.Δ5', pWE/AAV.Δ5', pWE/Ad-H.

20.   A recombinant nucleic acid comprising:
      adenovirus derived nucleotides 1-454 and adenovirus nucleotides 3511-6095 shown in Figures 21 and 22.

21.   A recombinant nucleic acid comprising:

20   a deletion in an E3 region of a recombinant nucleic deposited at the ECACC under No. P97082122, No. P97082119, No. P97082120, No. P97082116.

22.   A recombinant nucleic acid according to claim 21, wherein said deletion comprises a gp19K region.

25   23.   A recombinant nucleic acid comprising:
      a nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises sufficient adenovirus sequences necessary for replication and capsid gene expression, wherein said nucleotide sequence

30   comprises a deletion of at least the E1 region and encapsulating signal of said adenovirus.

24.   A recombinant nucleic acid comprising:
      a nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises

35   sufficient adenovirus sequences necessary for replication and capsid gene expression, and a complementary sequence to an

upstream part of the same strand of said nucleic acid,
wherein said complementary sequence can base-pair with said
upstream part so that it functions as a start-site for a
nucleic acid polymerase, wherein said nucleotide sequence
5   comprises a deletion of one Inverted Terminal Repeat, the E1
region and the encapsulating signal of said adenovirus.
    25.   A recombinant nucleic acid comprising:
            a nucleotide sequence based on or derived from an
adenovirus, wherein said nucleotide sequence comprises a
10 sequence for adenovirus-independent replication, and
sufficient adenoviral sequences necessary for replication,
wherein said nucleotide sequence comprises at least a
deletion of the E1 region and encapsulating signal of said
adenovirus.
15 26.   A recombinant nucleic acid according to claim 25,
wherein said nucleotide sequence further comprises a deletion
of at least one of the Inverted Terminal Repeats of said
adenovirus.
    27.   A recombinant nucleic acid according to claim 25 or
20 claim 26, wherein said sequence for adenovirus-independent
replication comprises an SV40 origin of replication.
    28.   A recombinant nucleic acid according to anyone of
claims 18-27 wherein said nucleotide sequence comprises no
sequences which allow for homologous recombination leading to
25 replication competent virus in a cell into which said
recombinant nucleic acid is transferred.
    29.   An adapter plasmid comprising:
            a nucleotide sequence based on or derived from an
adenovirus, wherein said nucleotide sequence comprises in
30 operable configuration at least one functional Inverted
Terminal Repeat, one functional encapsulating signal and
adenoviral sequences which allow for homologous recombination
and the generation of a replication-defective, recombinant
adenovirus genome.
35 30.   An adapter plasmid according to claim 29, comprising
no sequences which allow for homologous recombination leading

to replication competent virus in a cell into which said
adapter plasmid is transferred.

31.   An adapter plasmid according to claim 30, having no
E1 region sequences.

5   32.   An adapter plasmid according to anyone of claims 29-
31, further comprising a nucleic acid of interest such as a
multiple cloning site and/or a transgene.

33.   A recombinant nucleic acid according to anyone of
claim 18-28 or an adapter plasmid according to anyone of

10   claims 29-32, wherein said transgene is operatively linked to
an E3 promoter.

34.   A method for the generation of recombinant adenovirus
having an E1 deletion and a gp19K deletion, comprising the
steps of:

15   growing a cell comprising adenovirus complementing
sequences transfected with

i) an adapter plasmid comprising a first nucleotide
sequence based on or derived from an adenovirus, wherein said
nucleotide sequence comprises in operable configuration one

20   functional Inverted Terminal Repeat, one functional
encapsulating signal and adenoviral sequences which allow for
homologous recombination leading to the generation of a
replication-defective, recombinant adenovirus genome in a
cell into which said adapter plasmid is transferred and

25   having no E1 region sequences, and

ii) a recombinant nucleic acid comprising at least
one second nucleotide sequence based on or derived from an
adenovirus, wherein said at least one second nucleotide
sequence comprises one Inverted Terminal Repeat and

30   sufficient adenovirus sequences for replication and a partial
overlap with said adapter plasmid, wherein said at least one
second nucleotide sequence comprises a deletion of at least
the E1 region, encapsulating signal and gp19K sequences; .
wherein said complementing sequences, said first nucleotide

35   sequence and said at least one second nucleotide sequence

have no overlapping sequences which allow for homologous
recombination leading to replication competent virus,
under conditions whereby recombinant adenovirus having an E1
deletion and a gp19K deletion is generated.

5   35.   A method according to claim 34, wherein said adapter
plasmid further comprises a first heterologous nucleotide
sequence inserted into said E1 region deletion and said
recombinant nucleic acid further comprises a second
heterologous nucleotide sequence inserted into said gp19K

10   region.

        36.   A method for the generation of recombinant
adenovirus, comprising the steps of:
        growing a cell comprising adenovirus complementing
sequences transfected with

15   i) a first recombinant nucleic acid comprising a
first nucleotide sequence based on or derived from an
adenovirus, wherein said first nucleotide sequence comprises
a functional encapsulating signal and two functional Inverted
Terminal Repeats or functional fragments or derivatives

20   thereof, and wherein said first recombinant nucleic acid has
no functional adenovirus genes and
                ii) a second recombinant nucleic acid comprising a
second nucleotide sequence based on or derived from an
adenovirus, wherein said second nucleotide sequence comprises

25   sufficient adenovirus sequences for replication, wherein said
second nucleotide sequence comprises a deletion of at least
the E1 region and encapsulating signal of said adenovirus;
        wherein, said complementing sequences, said first
nucleotide sequence and said second nucleotide sequence have

30   no overlapping sequences which allow for homologous
recombination leading to replication competent virus,
        under conditions whereby recombinant adenovirus is
generated.

        37.   A method for the generation of recombinant

35   adenovirus, comprising the steps of:

growing a cell comprising adenovirus complementing
sequences transfected with
i) a first recombinant nucleic acid comprising a
first nucleotide sequence based on or derived from an
5   adenovirus, wherein said first nucleotide sequence comprises
a functional encapsulating signal and two functional Inverted
Terminal Repeats or functional fragments or derivatives
thereof, and wherein said first recombinant nucleic acid has
no functional adenovirus genes and
10   ii) a second recombinant nucleic acid comprising a
second nucleotide sequence based on or derived from an
adenovirus, wherein said second nucleotide sequence comprises
a sequence for adenovirus-independent replication, and
sufficient adenoviral sequences necessary for replication,
15   wherein said second nucleotide sequence comprises at least a
deletion of the E1 region and encapsulating signal of said
adenovirus;
wherein, said complementing sequences, said first
nucleotide sequence and said second nucleotide sequence have
20   no overlapping sequences which allow for homologous
recombination leading to replication competent virus,
under conditions whereby recombinant adenovirus is
generated.
38.   A method according to claim 37, wherein said cell
25   comprises at least one nucleic acid molecule whereby said
cell expresses SV40 Large T antigen proteins or functional
fragments thereof.
39.   A method according to claim 37 or claim 38, wherein
said second recombinant nucleic acid molecule is replicated.
30   40.   A replication defective adenovirus comprising:
a genome based on or derived from an adenovirus,
wherein said genome comprises at least a functional
encapsulating signal and two functional Inverted Terminal
Repeats or functional fragments or derivatives thereof and
35   wherein said genome comprises no functional adenovirus genes
and has no overlapping sequences which allow for homologous

recombination leading to replication competent virus in a
cell into which said replication defective adenovirus is
transferred.

41   A replication defective adenovirus according to claim
40, further comprising:

one or more nucleic acids of interest.

42.   A non-human cell comprising a genome of a replication
defective adenovirus according to claim 40 or claim 41..

43.   A non-human cell according to claim 42, wherein said
cell is a mammalian cell.

44.   A method for transducing a cell, comprising the step
of:

contacting said cell with a replication defective
adenovirus according to claim 40 or claim 41 under conditions
whereby said cell is transduced.

45.   A non-human cell produced according to the method of
claim 44, wherein said cell is a mammalian cell.

46.   A method for generating recombinant adenovirus
comprising the step of:

growing a cell comprising adenovirus complementing
sequences and

i) a first recombinant nucleic acid comprising a
first nucleotide sequence based on or derived from an
adenovirus, wherein said first nucleotide sequence comprises
a functional encapsulating signal and two functional Inverted
Terminal Repeats or functional fragments or derivatives
thereof, and wherein said first recombinant nucleic acid has
no functional adenovirus genes and,

ii) a second recombinant nucleic acid comprising a
second nucleotide sequence based on or derived from an
adenovirus, wherein said nucleotide sequence comprises at
least all adenovirus sequences, or functional fragments or
derivatives thereof necessary for replication and capsid gene
expression, and a complementary sequence to an upstream part
of the same strand of said nucleic acid, wherein said

complementary sequence can base-pair with said upstream part
so that it functions as a start-site for a nucleic acid
polymerase, wherein said second nucleotide sequence comprises
a deletion of one Inverted Terminal Repeat, the E1 region and
5   the encapsulating signal of said adenovirus;
            wherein, said complementing sequences, said first
nucleotide sequence and said second nucleotide sequence have
no overlapping sequences which allow for homologous
recombination leading to replication competent virus,
10   under conditions whereby recombinant adenovirus is
generated.
    47.   A cell comprising a recombinant nucleic acid
according to any one of claims 18-28, 33 or a replication
defective adenovirus vector according to anyone of claims 40,
15   41, 55 or 56 and/or an adapter plasmid according to anyone of
claims 29-32.
    48.   A method for the replacement of a defective gene in a
host cell genome comprising the step of:
            growing said host cell with a recombinant nucleic
20   acid molecule derived from a replication defective adenovirus
comprising a functional version or part thereof of said
defective gene under conditions whereby at least one allele
of said defective gene in said host cell genome is replaced.
    49.   A method according to claim 44, wherein said
25   replication defective adenovirus expresses no adenoviral
genes.
    50.   A method according to claim 44, wherein said-
defective gene is a defective tumor suppressor gene.
    51.   An isolated cell comprising a genome of a
30   replication-defective adenovirus according to any one of
claims 40, 41, 55 or 56.
    52.   A isolated cell according to claim 51, wherein said
cell is a human cell.
    53.   A recombinant nucleic acid according to anyone of
35   claims 18-28, wherein said deletion in the E3 region is
replaced with a transgene.

54.   A method according to claim 34 or claim 35, wherein said at least one second nucleotide sequence comprises a first and second molecule wherein said first molecule has said partial overlap with said adapter plasmid at the 3' end,

5   and said second molecule comprises said Inverted Terminal Repeat and region including deletion of said gp19K sequences.

55.   A replication-defective adenovirus comprising:
a genome based on or derived from an adenovirus, wherein said genome comprises a first deletion. in and E1 region and a

10   second deletion in a gp19K region.

56.   A replication-defective adenovirus according to claim 55, wherein transcription of said transgene is directed by an E3 promoter.

57.   An isolated cell comprising:

15   a recombinant nucleic acid according to any one of claims 18-28, 33 or a replication defective adenovirus vector according to anyone of claims 40, 41, 55 or 56 and/or an adapter plasmid according to anyone of claims 29-32.

58.   An isolated cell according to claim 57, wherein said

20   cell is a human cell.
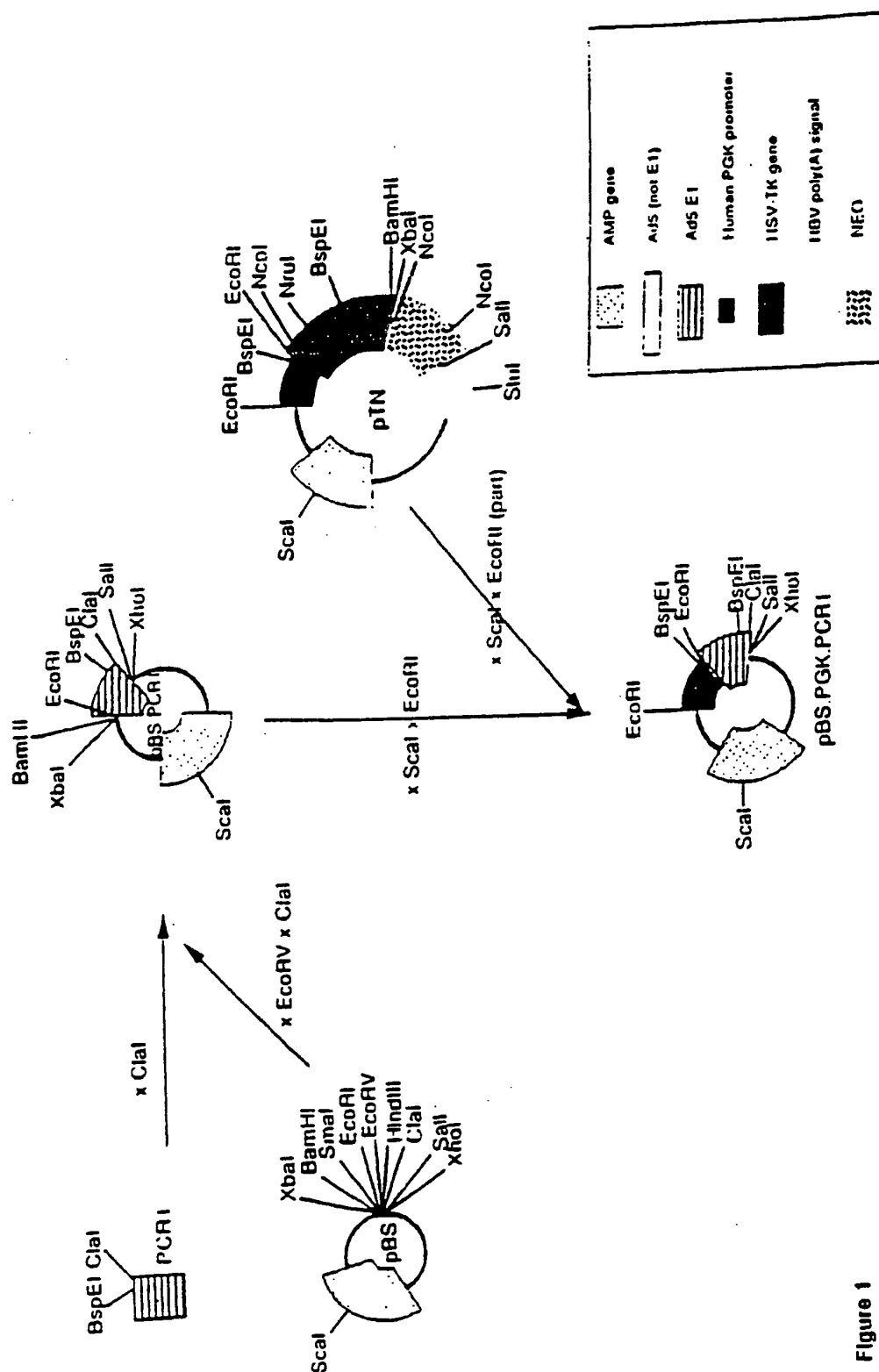
Figure 1

Construction of pBS.PGK.PCRI
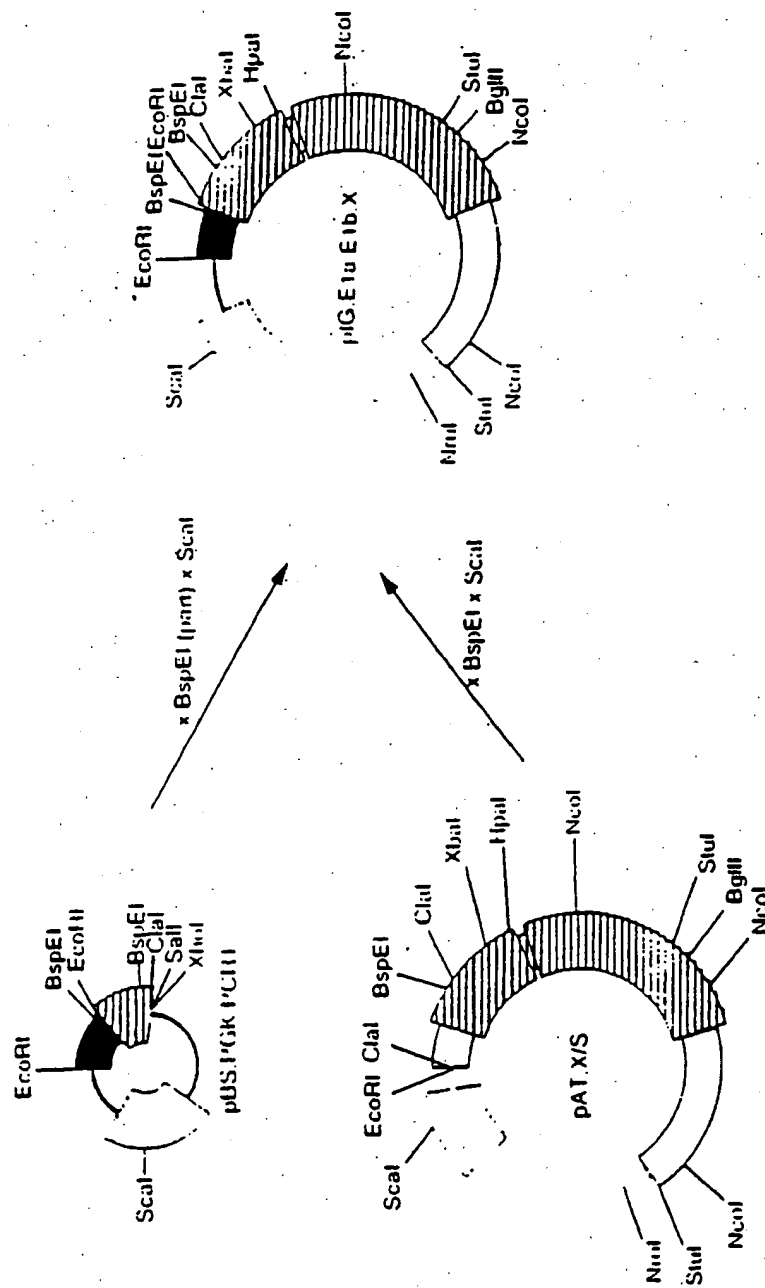
Figure 2

Construction of pIG.E1a.E1b.X
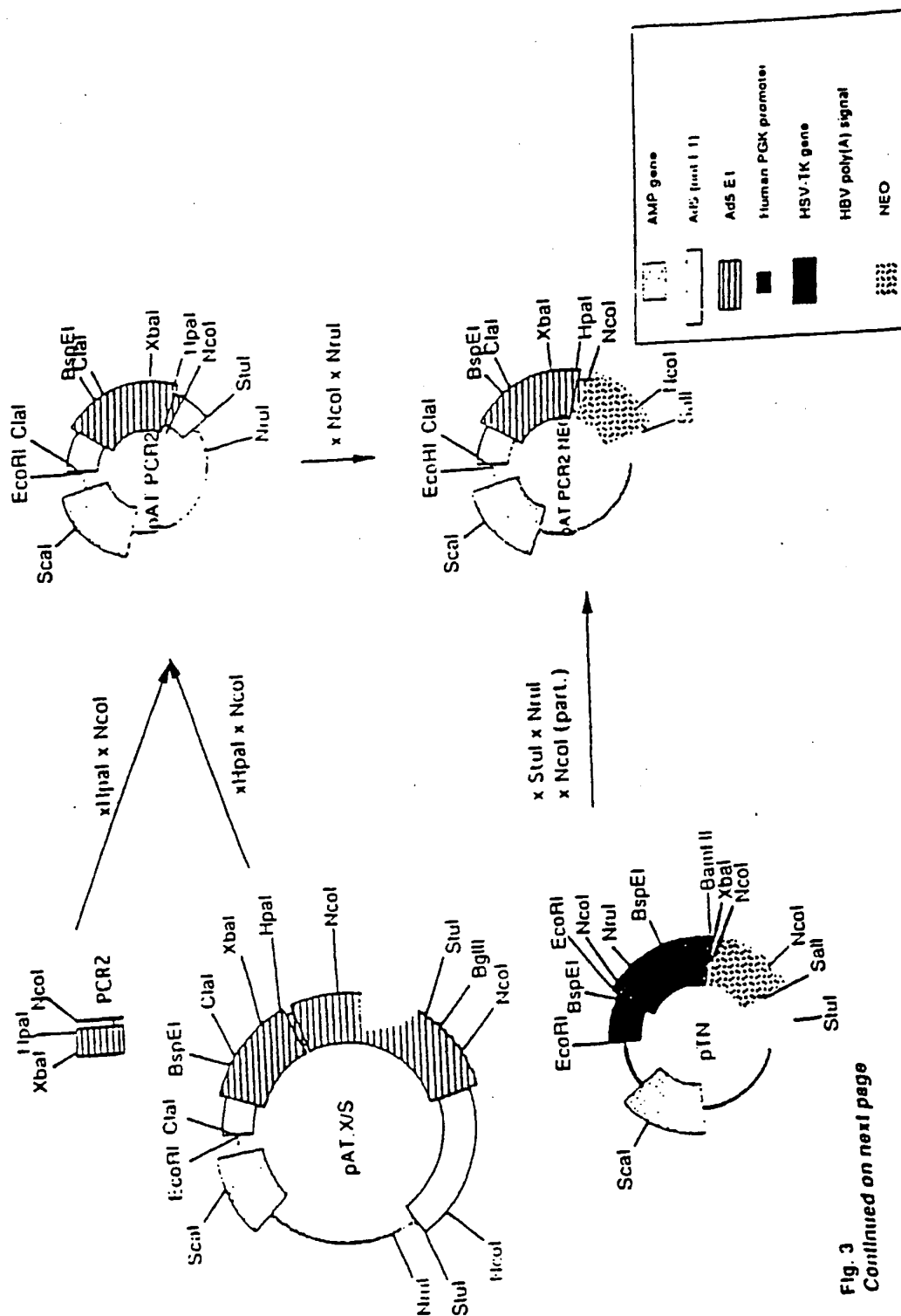
Fig. 3
Continued on next page
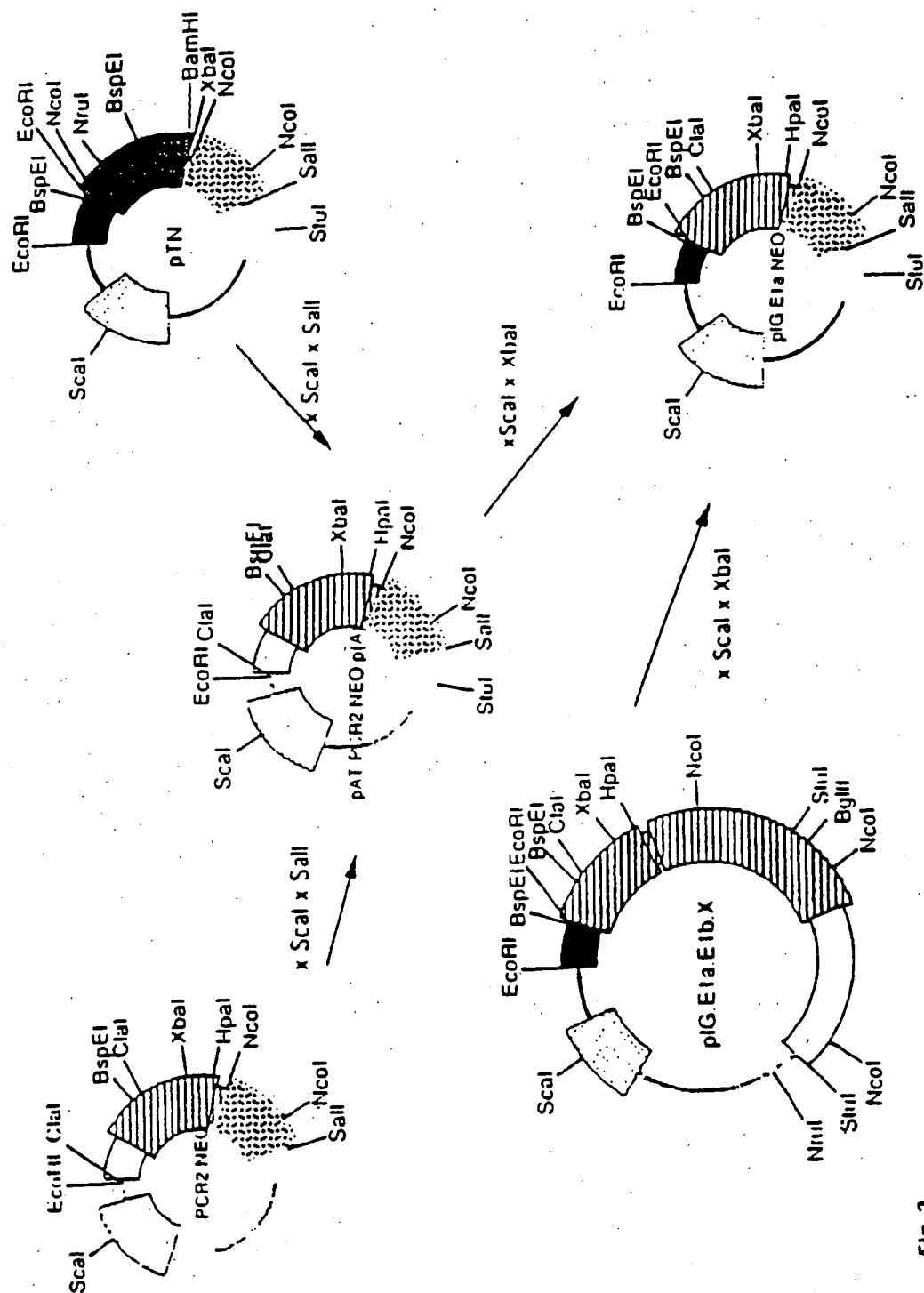
Fig. 3.
Construction of pIG.E1B.NEO
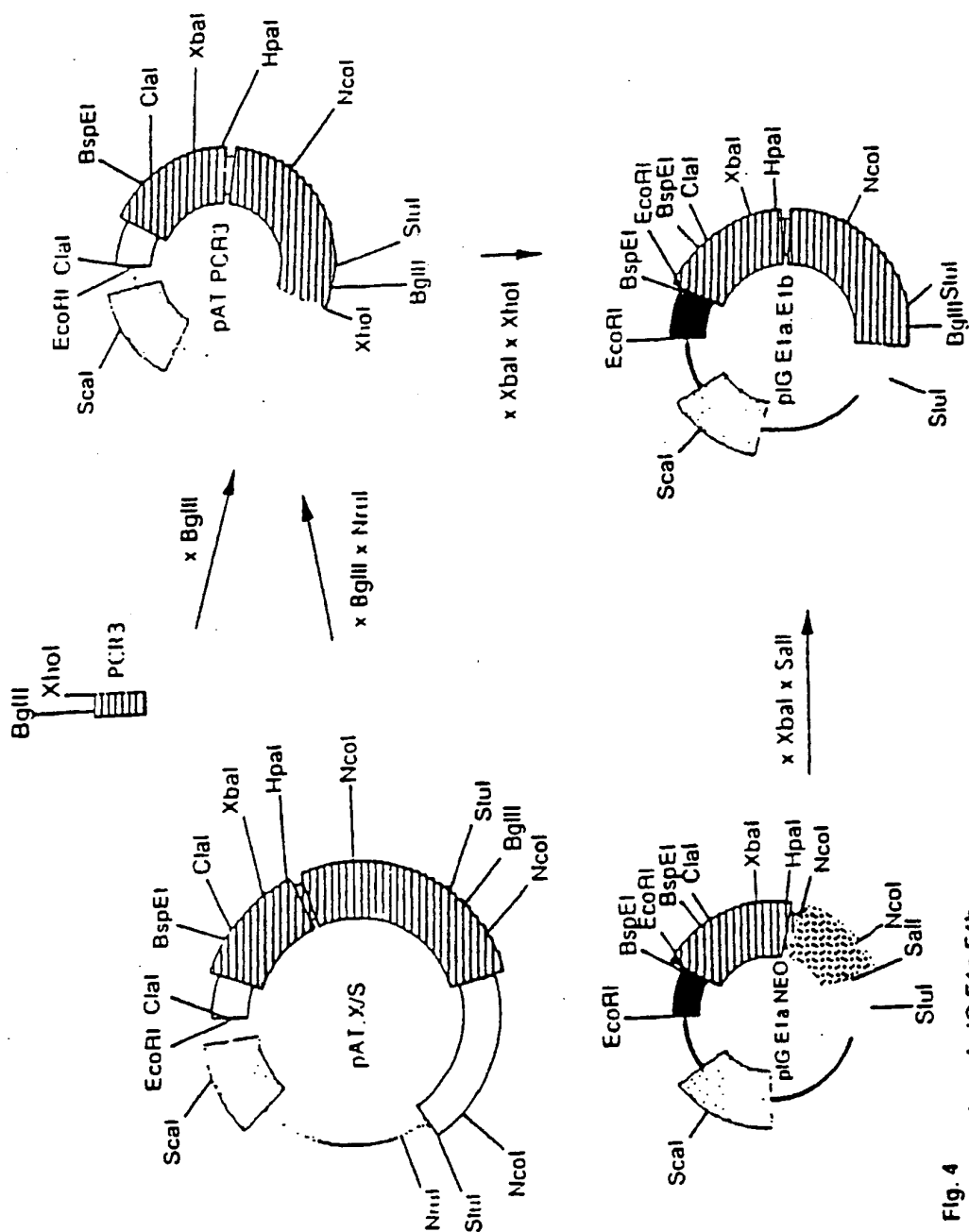
Fig. 4
Construction of pIG.E1a.E1b

Fig. 4
Construction of pIG.E1a.E1b

Fig. 5
Construction of pIG.NEO

Figure 6
Overview of available adenovirus packaging constructs and assessment of their capacity to transform primary kidney cells

Figure 7

Western blotting analysis of A549 clones transfected with pIG.E1A.NEO and PER clones (HER cells transfected with pIG.E1A.E1B)
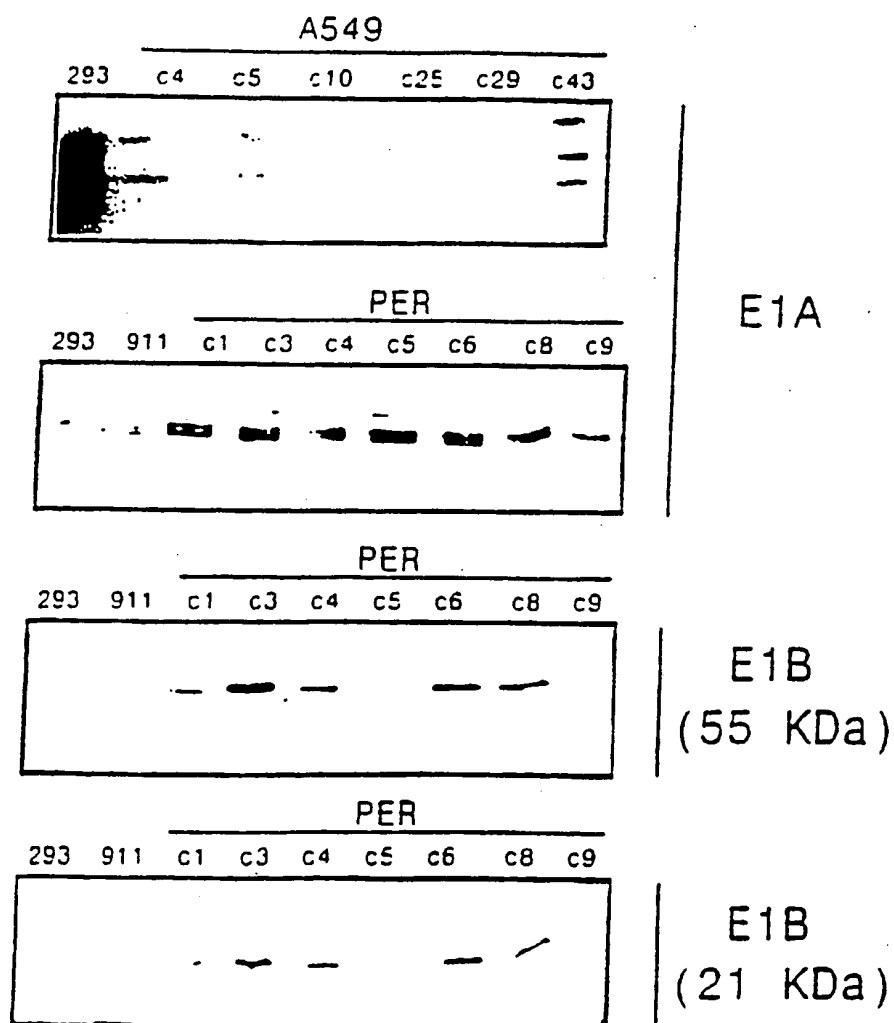
Figure 8

Southern blot analyses of 293, 911 and PER cell lines
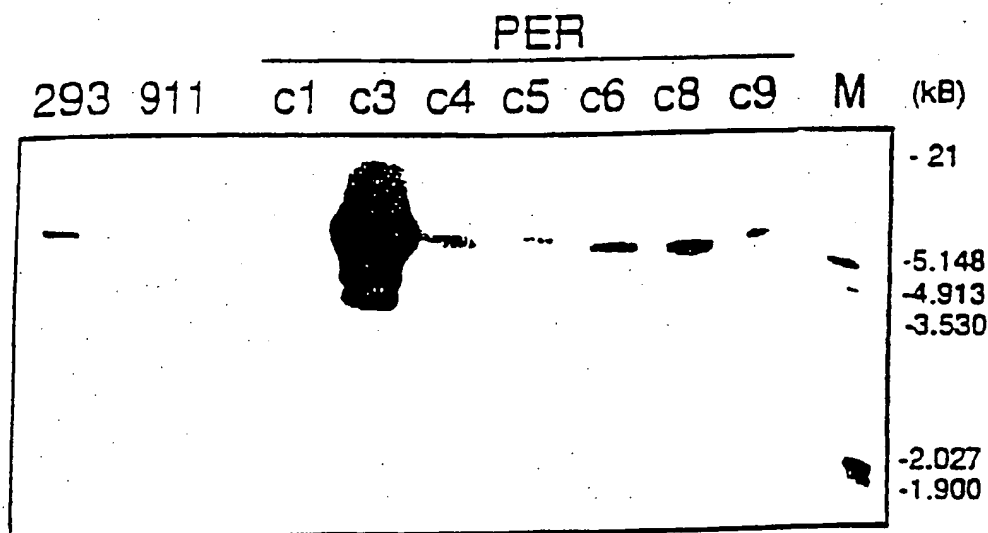
Figure 9

Transfection efficiency of PER.C3. PER.C5. PER.C6 and 911 cells.
Cells were cultured in 6-well plates and transfected (n=2) with 5 μg
pRSV.lacZ by calcium-phosphate co-precipitation. Forty-eight hours later
the cells were stained with X-GAL. The mean percentage of blue cells is
shown.
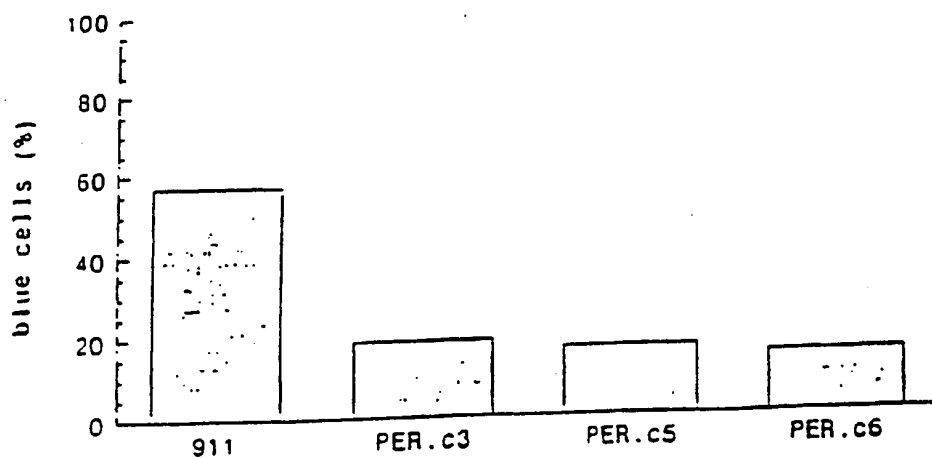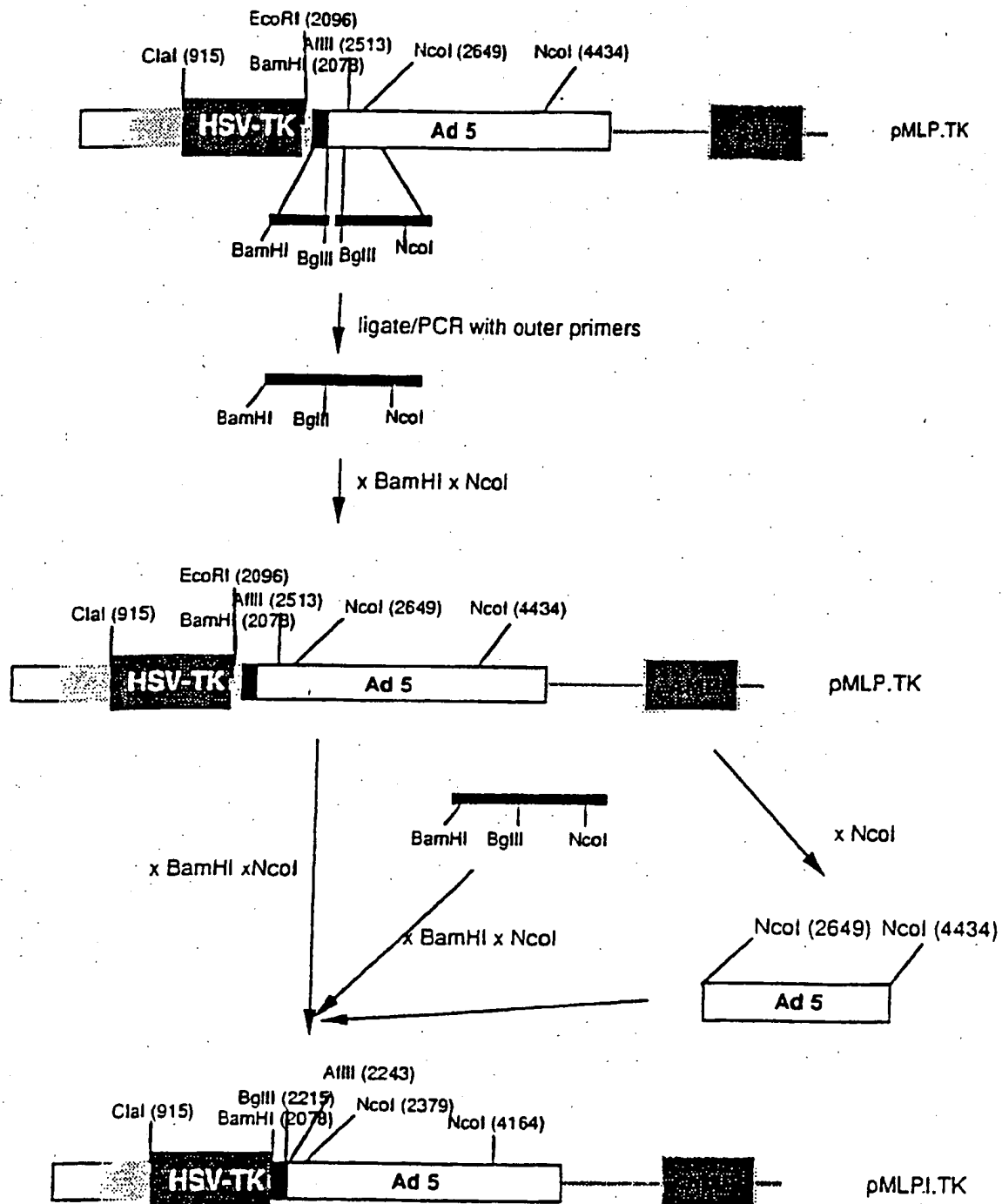
Fig. 10

New recombinant adenoviruses and packaging constructs without sequence overlap
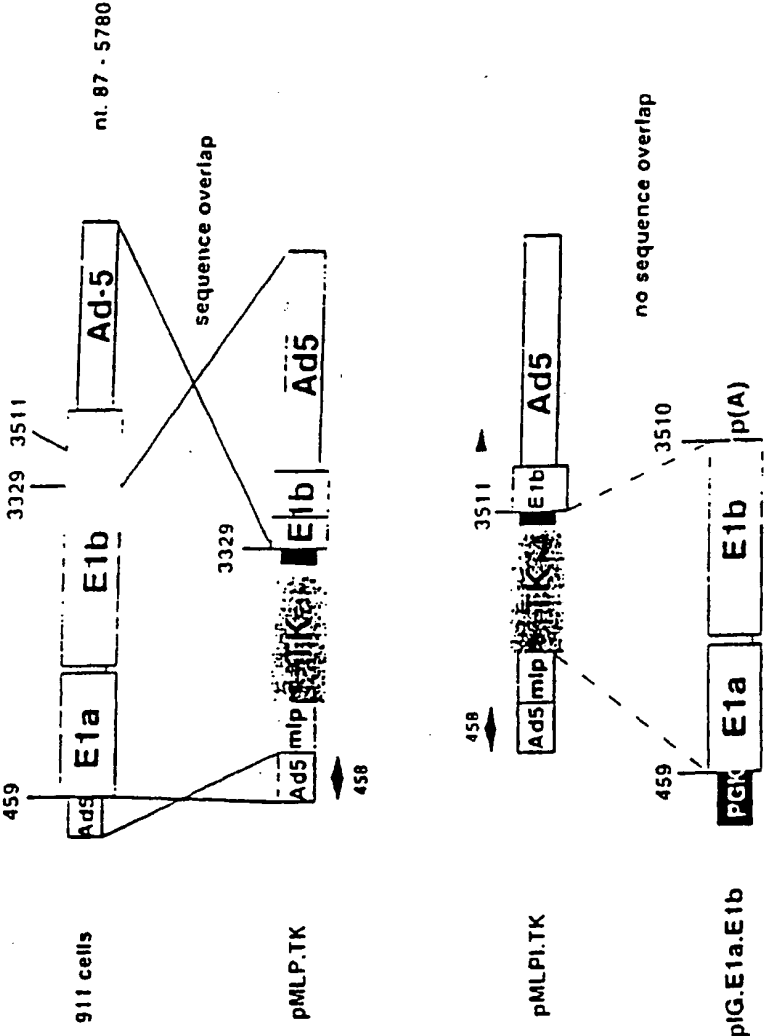
Figure 11a
·Packaging system based on primary cells

New recombinant adenoviruses and packaging constructs without sequence overlap
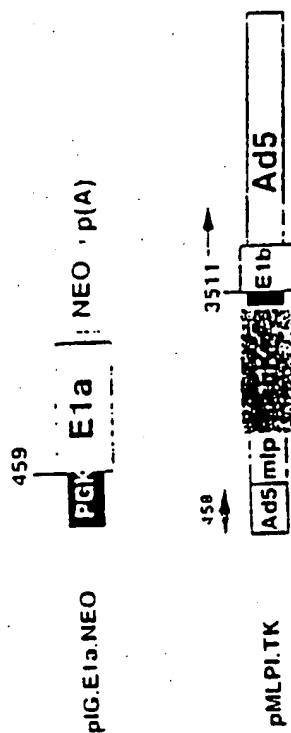
Figure 11.b
Packaging system based on established cell lines: transfection with E1a and selection with G418

Generation of recombinant adenovirus
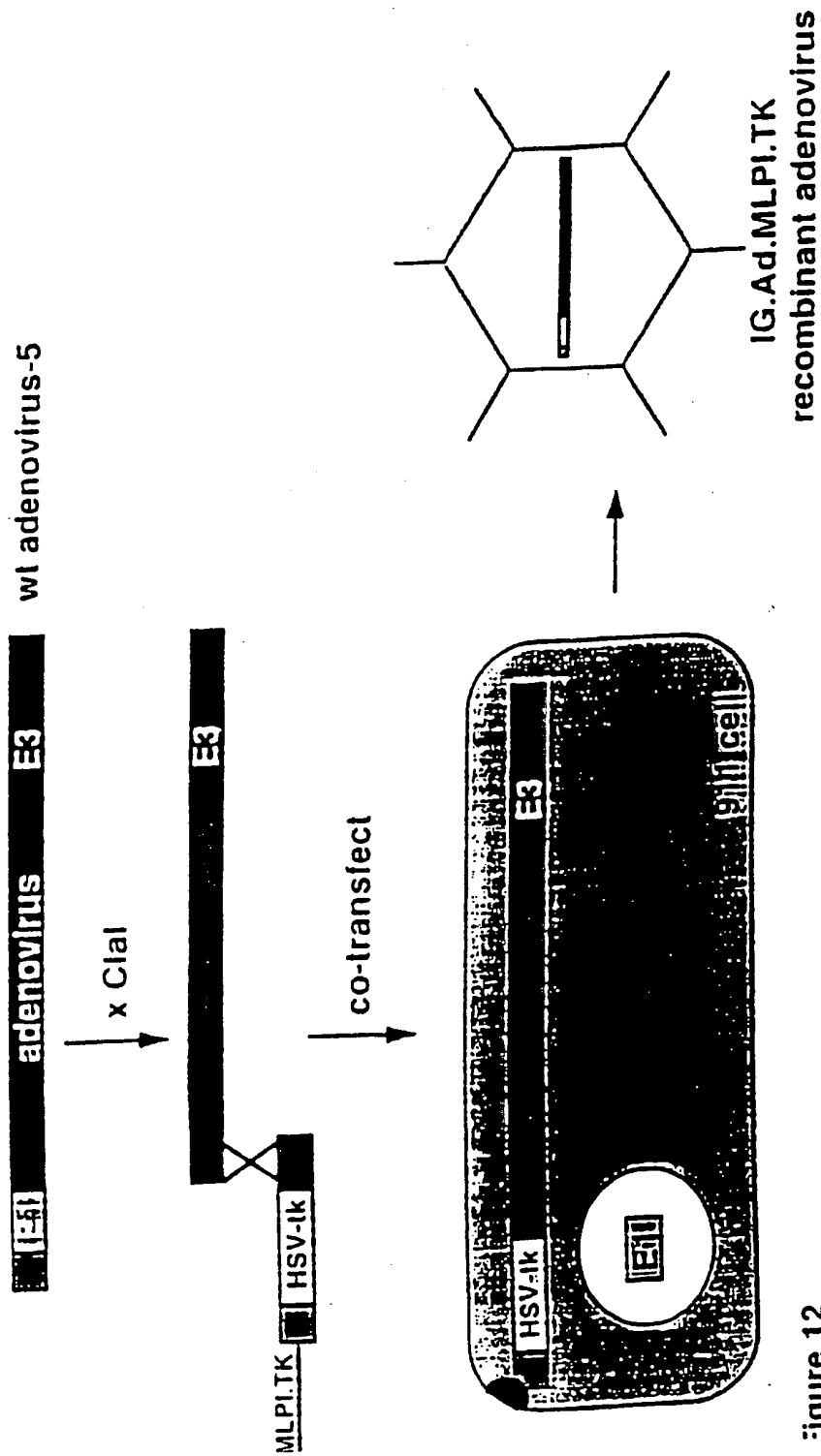
Figure 12
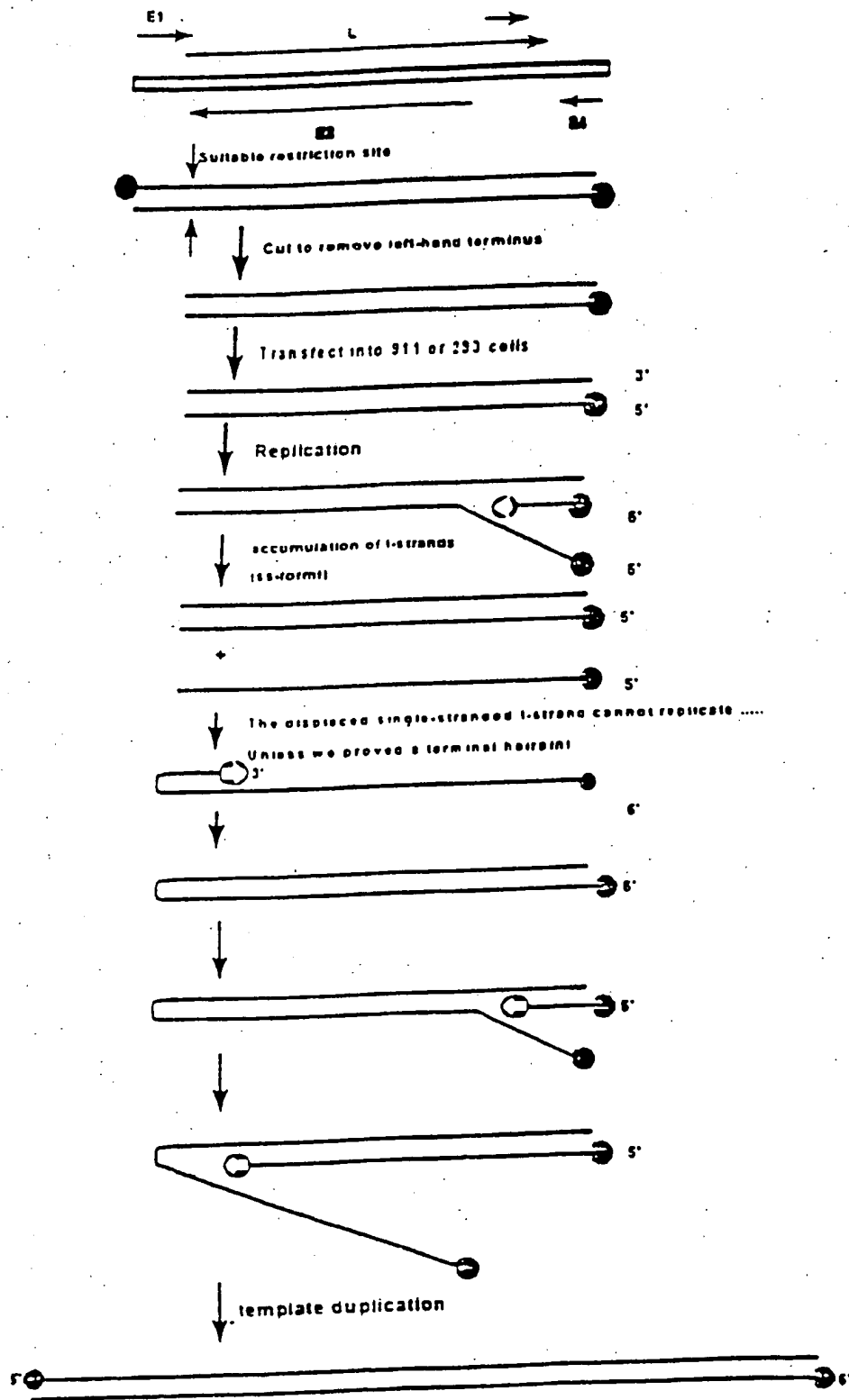
Figu   13

Replication of Adenovirus
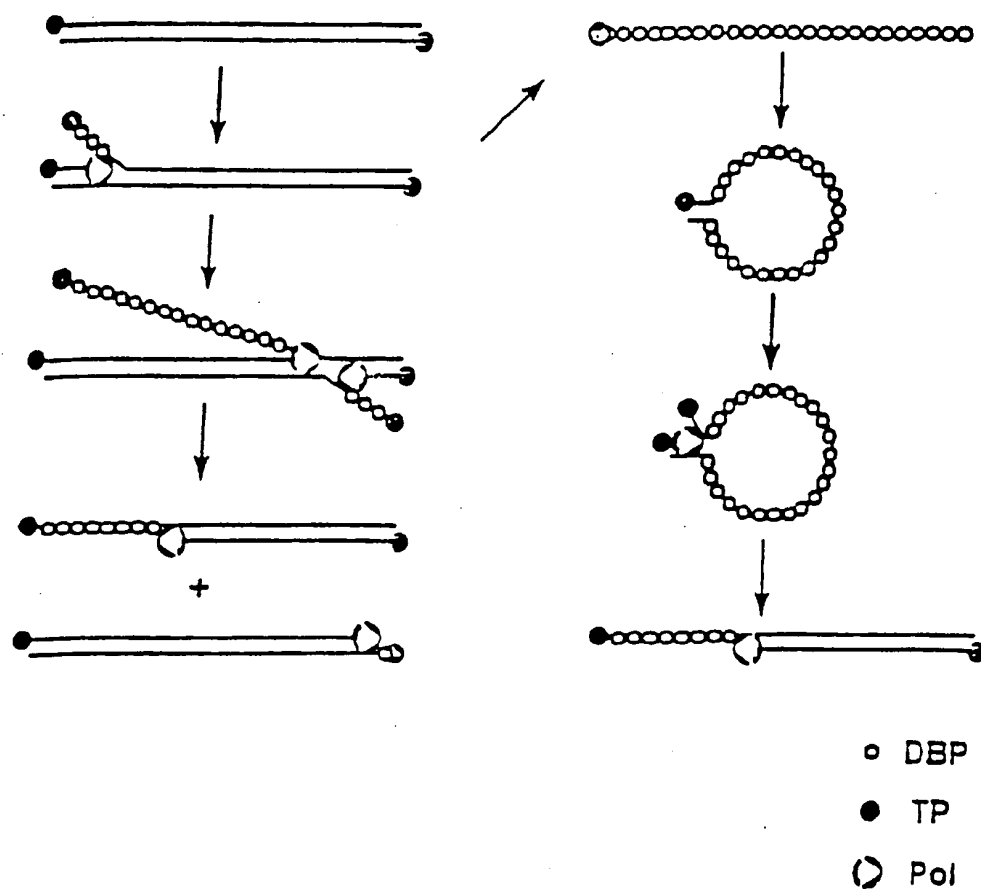
Figure 14

```
5'-GTACACTGACCTAGTGCCGCCCGGCA
           | | | | | | | | | | | | | | | | | A
     3'-GATCACGGCGGGCCCA
```

Fig 15. The potential hairpin conformation of a single-stranded
DNA molecule that contains the HP/asp sequences used in these
studies. Restriction with the restriction endonuclease Asp718I
of plasmid pICLha., containing the annealed oligonucleotide pair
HP/asp1 en HP/asp2 will yield a linear double-stranded DNA
5 fragment. In cells in which the required adenovirus genes are
present, replication can initiate at the terminus that contains
the ITR sequence. During the chain elongation, the one of the
strands will be displaced. The terminus of the single-stranded
displaced- strand molecule can adopt the conformation depicted
10 above. In this conformation the free 3'-terminus can serve as a
primer for the cellular and/or adenovirus DNA polymerase,
resulting in conversion of the displaced strand in a double-
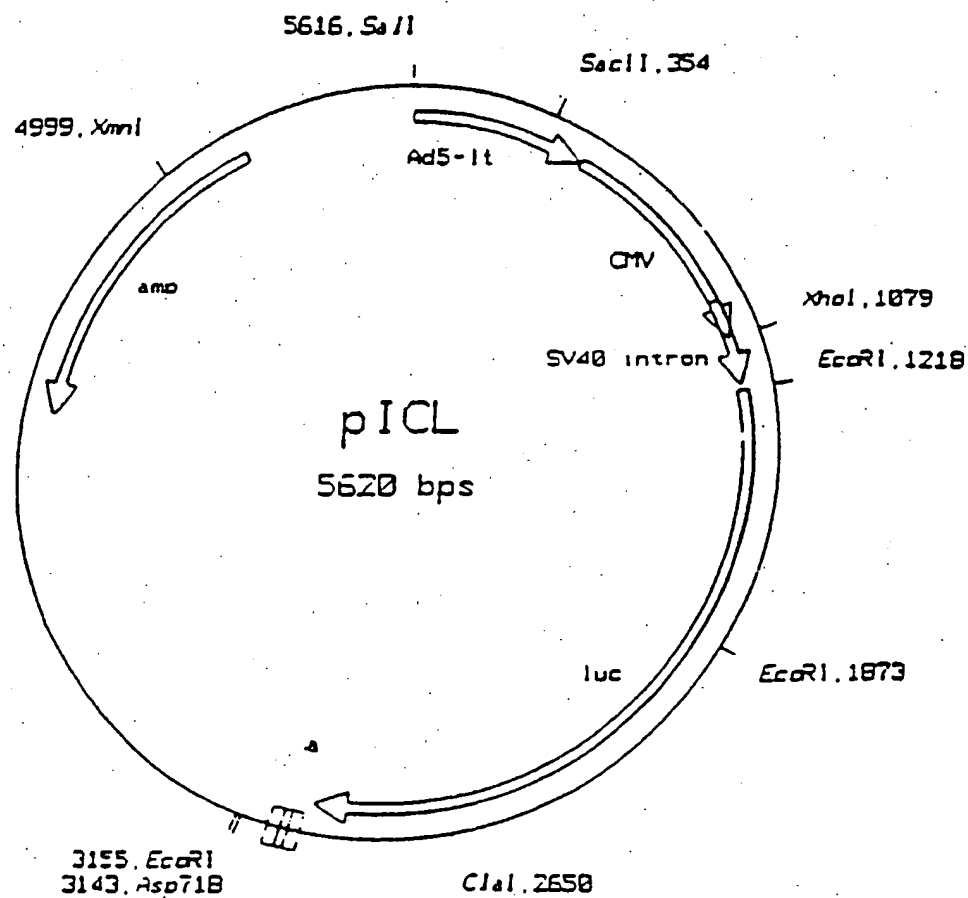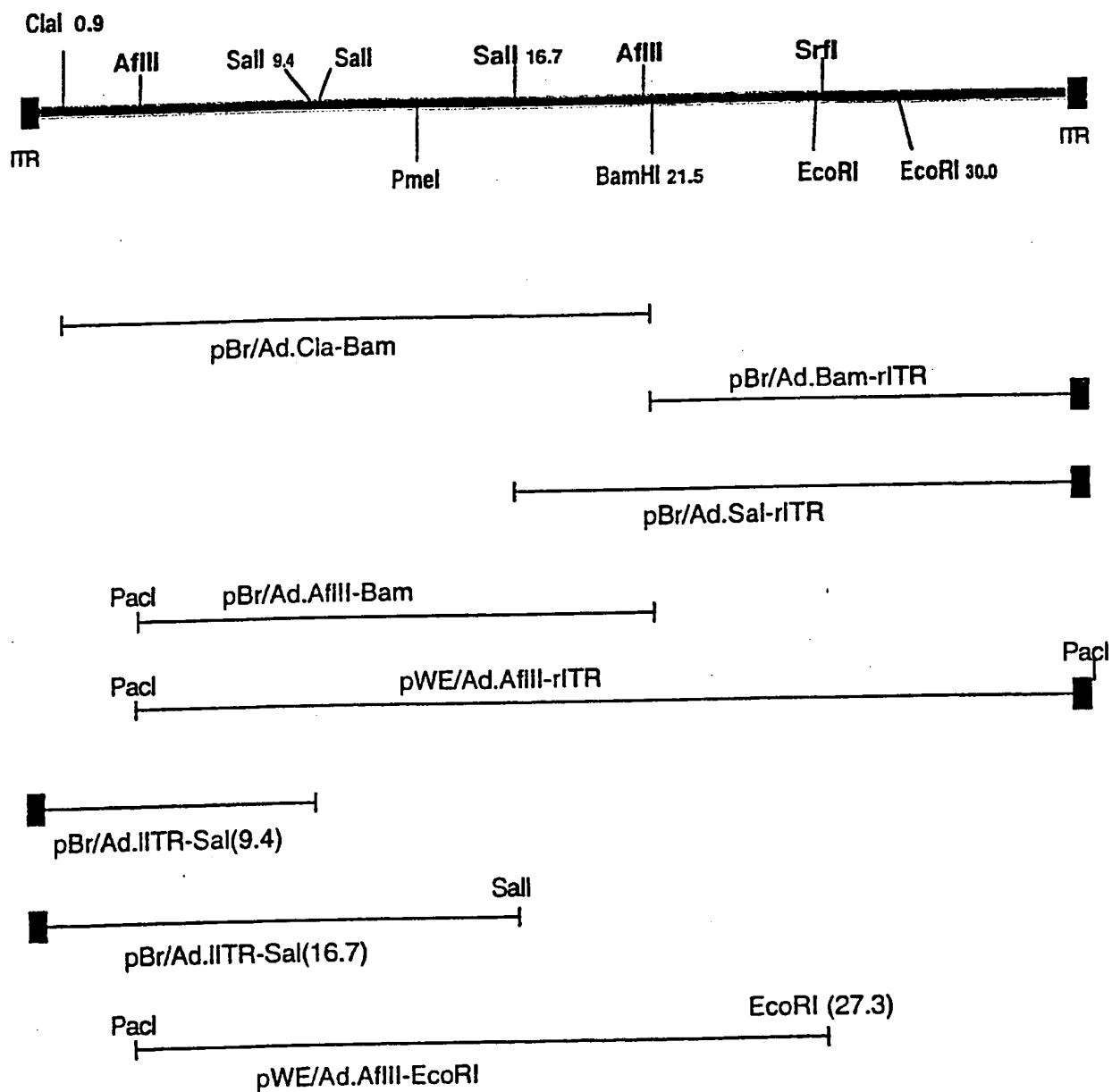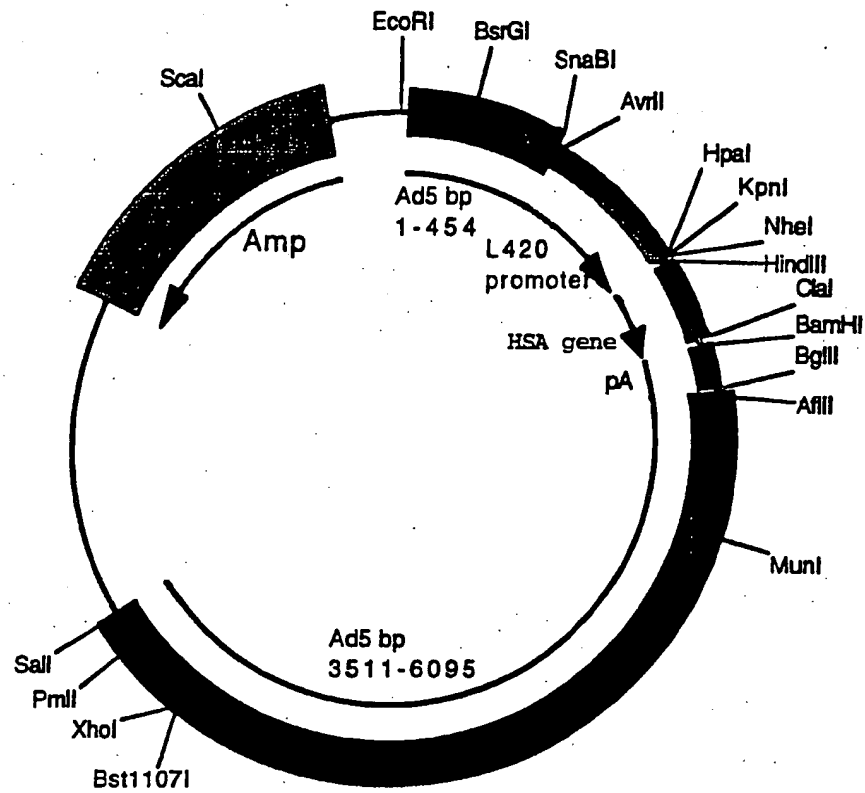stranded form.

Figure 15
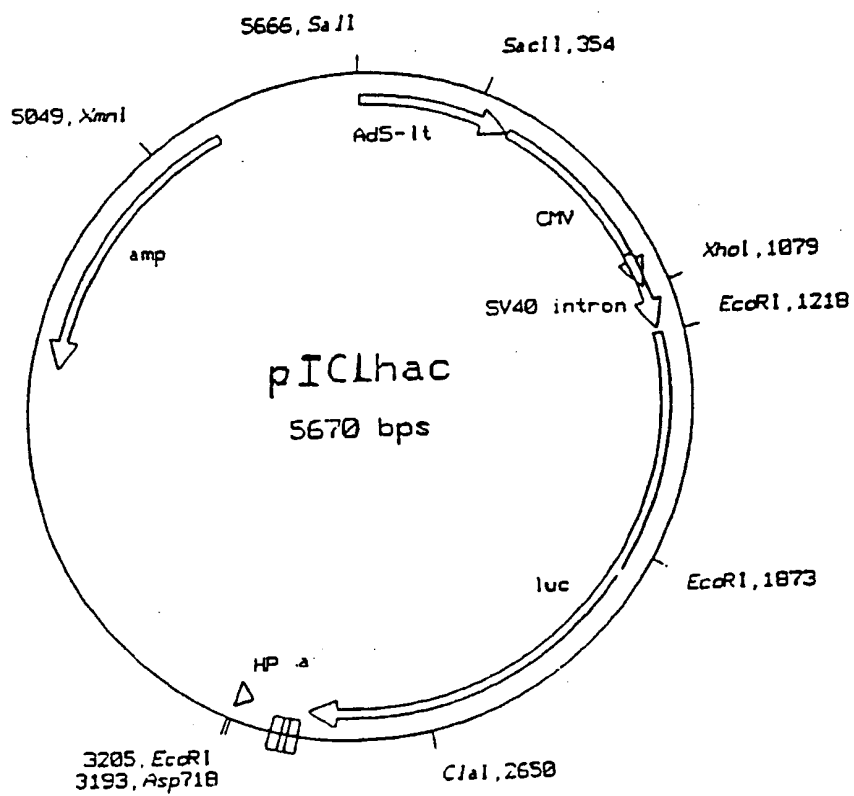
Fig. 16
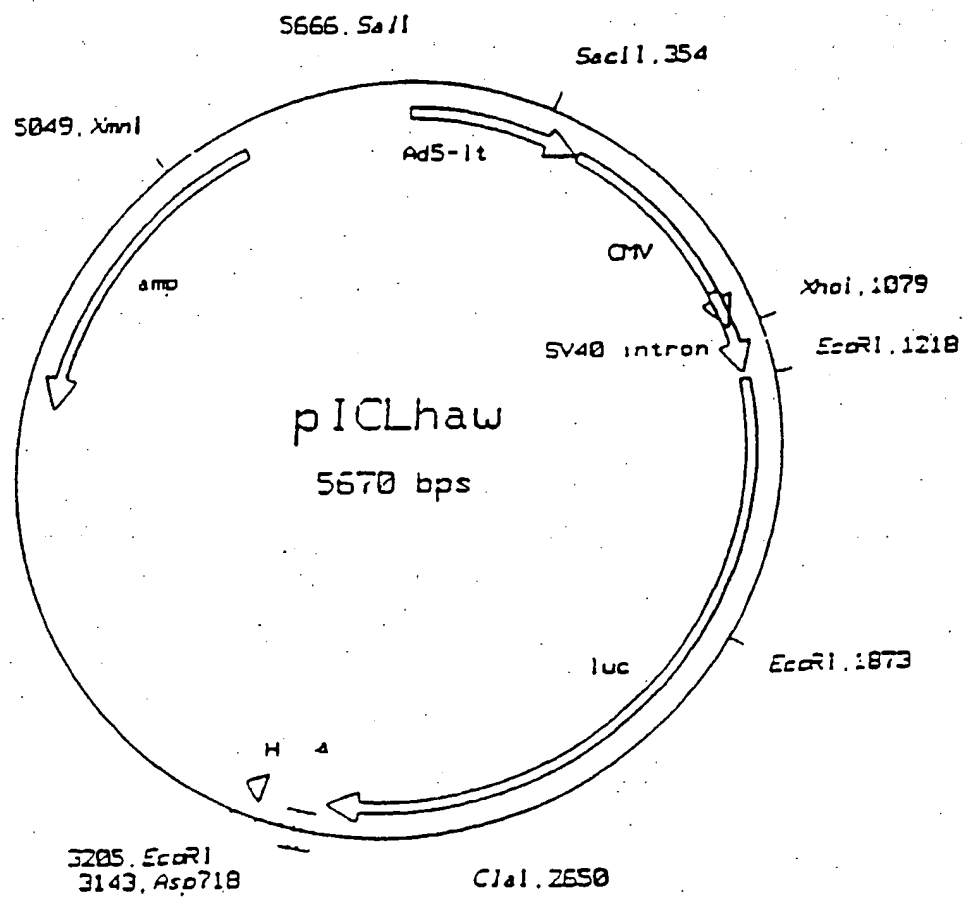
Figure 17
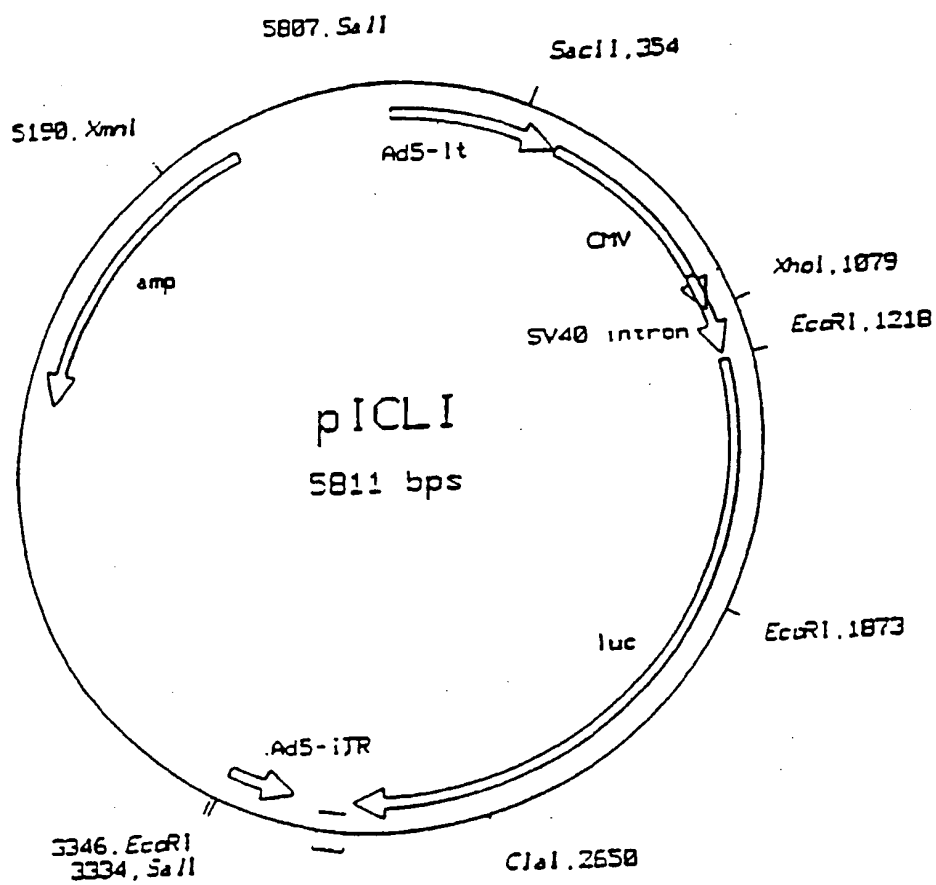
Figure 18

Figure 19

Figure 20: Cloned adenovirus fragments

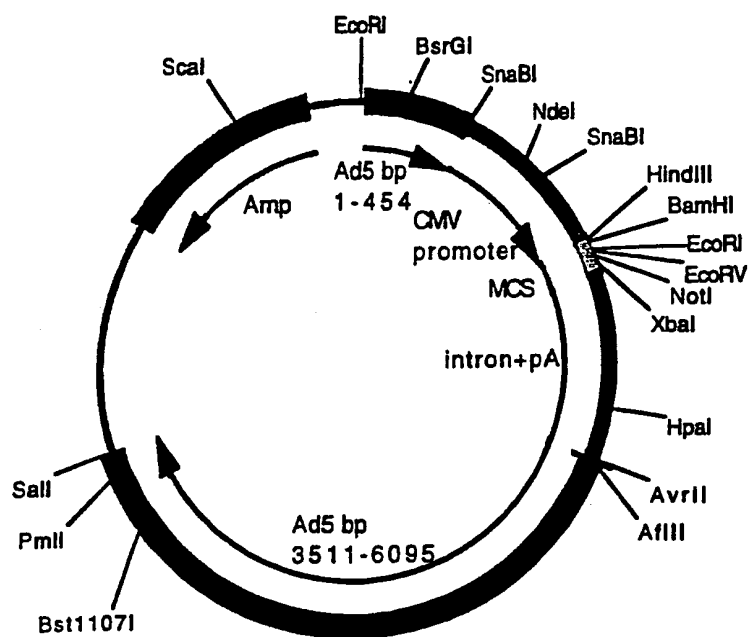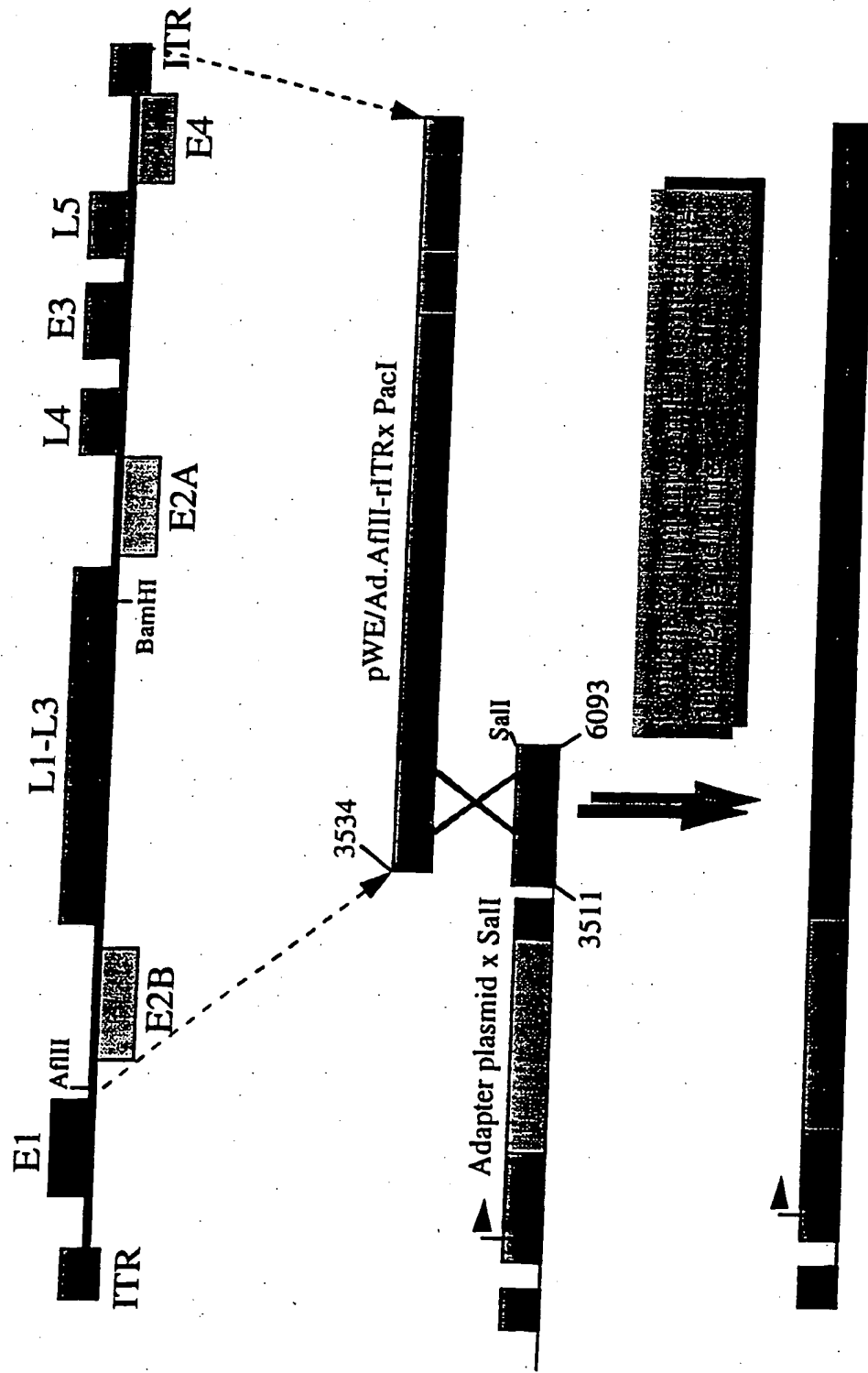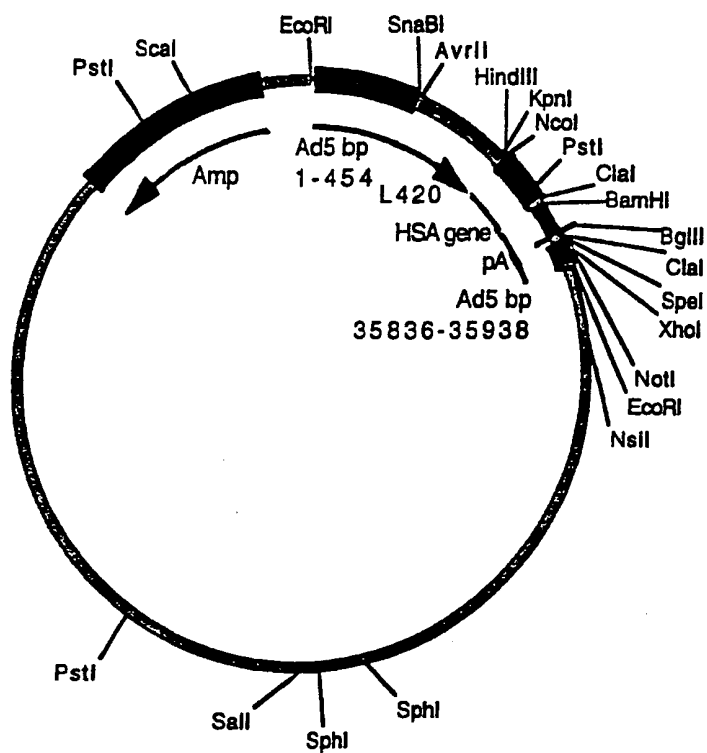## Figure 21: Adapter plasmid pAd5/L420-HSA

Figure 22: Adapter plasmid pAd5/CLIP
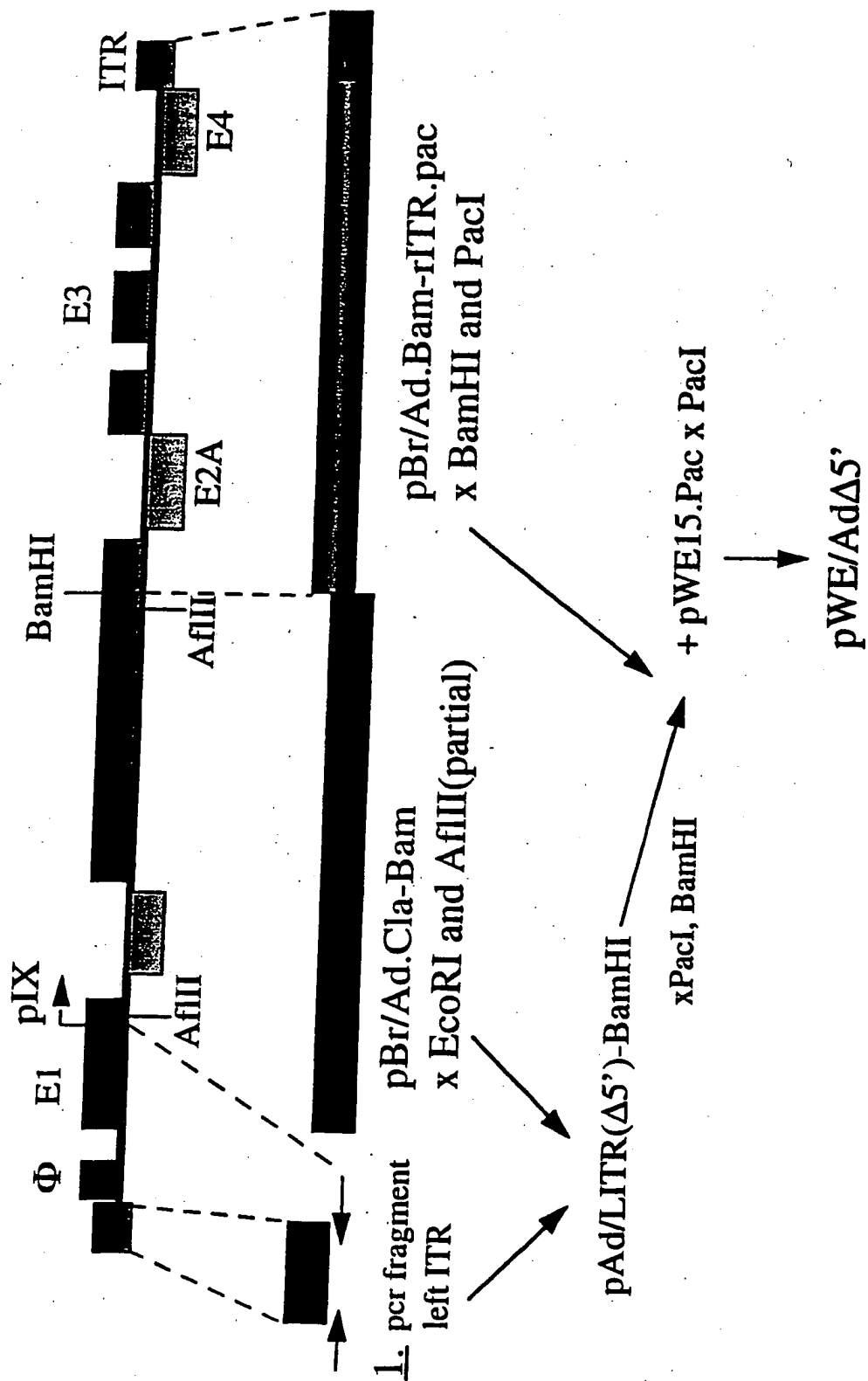
Figure 23: Generation of recombinant adenoviruses

Figure 24: Minimal adenovirus vector pMV/L420H

Figure 25: Construction of pWE/AdΔ5'

Figure 26

A

pWE/AdΔ5'
(40695 bp)

Mbol (39369)
Mbol (38482)
Pacl (1)
BsrGl (4741)
Sall (34980)
Sall (6077)
pWE probe
Sall (6456)
SV40 ori
Pacl (32577)
Sall (13361)
EcoRl (26664)
Adeno probe
BsrGl
BsrGl (16313)
EcoRl (23946)
BsrGl
BamHl (18177)

B

21226
5148
4268
2027
1904
1584
1375
947
831
564

C

21226
5148
4268
2027
1904
1584
1375
947
831